

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:)	Examiner: Wegert, Sandra
)	
GODDARD, et al.)	Art Unit: 1647
)	
Application Serial No. 09/997,529)	Confirmation No: 1269
)	
Filed: November 15, 2001)	Attorney's Docket No. 39780-2730 P1C33
)	
For: SECRETED AND)	Customer No. 35489
TRANSMEMBRANE)	
POLYPEPTIDES AND NUCLEIC)	
ACIDS ENCODING THE SAME)	

FILED VIA EFS
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ON APPEAL TO THE BOARD OF PATENT APPEALS AND INTERFERENCES

APPELLANTS' BRIEF

MAIL STOP APPEAL BRIEF - PATENTS

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

On April 12, 2006, the Examiner made a Final Rejection to pending Claims 119-126, 129-131. A Final response was filed August 10, 2006 and a Notice of Appeal was filed on October 3, 2006. An Advisory Action was mailed on November 1, 2006.

Appellants hereby appeal to the Board of Patent Appeals and Interferences from the last decision of the Examiner.

The following constitutes Appellants' Brief on Appeal.

1. REAL PARTY IN INTEREST

The real party in interest is Genentech, Inc., South San Francisco, California, by an assignment of the parent application, U.S. Patent Application Serial No. 09/941,992 recorded November 16, 2001, at Reel 012176 and Frame 0450.

2. RELATED APPEALS AND INTERFERENCES

The claims pending in the current application are directed to polypeptides referred to herein as "PRO1182." Related patent applications, U.S. Serial No. 09/998,041, filed November 15, 2001 (containing claims directed to the PRO1182 antibodies), and U.S. Serial No. 09/989,734, filed November 19, 2001 (containing claims directed to the PRO1182 nucleic acids) are also under final rejection from the same Examiner, and based upon the same outstanding rejection(s), appeals of the final rejection are being pursued independently and concurrently herewith.

3. STATUS OF CLAIMS

Claims 122-126, 129-131 are in this application.

Claims 1-118, 127-128 are canceled. Further, Claims 119-121 were canceled, without prejudice, in the Response to final filed August 10, 2006. This amendment was entered by the Examiner, as indicated in the Advisory Action filed November 1, 2006.

Thus, Claims 122-126, 129-131 stand rejected and Appellants appeal the rejection of these claims.

A copy of the rejected claims involved in the present Appeal is provided in the Claims Appendix.

4. STATUS OF AMENDMENTS

The amendments submitted after final rejection have been entered, as indicated in the Advisory Action filed November 1, 2006.

5. SUMMARY OF CLAIMED SUBJECT MATTER

The invention claimed in the present application is related to an isolated polypeptide comprising the amino acid sequence of the polypeptide of SEQ ID NO:357, referred to in the present application as "PRO1182." PRO1182 was shown for the first time in the present application to inhibit the uptake of glucose or FFA (free fatty acids) by adipocyte cells (Example 158). This feature is specifically recited in Claim 124, and is carried by all claims dependent from Claim 124. In addition, the invention also claims the amino acid sequence of the polypeptide of SEQ ID NO:357, lacking its associated signal-peptide; or the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 203088 (Claims 124, 126 and 129). The invention is further directed to polypeptides having at least 95% to 99% amino acid sequence identity to the amino acid sequence of the polypeptide of SEQ ID NO:357; the amino acid sequence of the polypeptide of SEQ ID NO:357, lacking its associated signal peptide; or the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 203088, wherein said polypeptide enhances the uptake of glucose or FFA (free fatty acids) by adipocyte cells (Claims 122-123). The invention is further directed to a chimeric polypeptide comprising one of the above polypeptides fused to a heterologous polypeptide (Claim 130), and to a chimeric polypeptide wherein the heterologous polypeptide is an epitope tag or an Fc region of an immunoglobulin (Claim 131). PRO1182 polypeptide variants having at least about 95-99% amino acid sequence identity with a full length PRO polypeptide sequence, or a PRO polypeptide sequence lacking the signal peptide are generally described in the specification at, for example, page 305, line 23 onwards, and percent amino acid sequence identity determination is generally described at least at, for example, pages 306-308, line 14 onwards. The preparation of chimeric PRO polypeptides (Claims 130 and 131), including those wherein the heterologous polypeptide is an epitope tag or an Fc region of an immunoglobulin, is set forth in the specification at page 374, lines 24 to page 375, line 9. Examples 140-143 and page 376, line 12 onwards describe the expression of PRO polypeptides in various host cells, including *E. coli*, mammalian cells, yeast and Baculovirus-infected insect cells.

The amino acid sequence of the "PRO1182" polypeptide and the nucleic acid sequence encoding this polypeptide (referred to in the present application as "DNA59848-1512") are shown in the present specification as SEQ ID NOs: 357 and 356, respectively, and in Figures 252 and 251, described on pages 300, lines 8-11. The full-length PRO1182 polypeptide having the

amino acid sequence of SEQ ID NO:357 is described in the specification at, for example, on page pages 226-228; and the isolation of cDNA clones encoding PRO1182 of SEQ ID NO:357 is described in Example 111, page 492 of the specification, and is described in Figure 251 and SEQ ID NO: 356. That PRO1182 is a polypeptide having homology to conglutinin protein, a member of the C-type lectin family, is described at pages 33-34 and page 364.

Finally, Example 158, in the specification at page 530, shows that PRO1182 tested positive in the Adipocyte Glucose/ FFA uptake assay, demonstrating that PRO1182 is active as a stimulator of glucose and/or FFA uptake, and therefore would have utility in the therapeutic treatment of disorders where the stimulation of glucose uptake by adipocytes would be beneficial, for example, obesity, diabetes or hyper- or hypo-insulinemia.

6. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

- I. Whether Claims 122-126, 129-131 satisfy the utility requirement of 35 U.S.C. §101 and 35 U.S.C. §112, first paragraph.
- II. Whether Claims 122-123 satisfy the written description requirement of 35 U.S.C. §112, first paragraph.

7. ARGUMENT

Summary of the Arguments

Issue I: Utility

The Examiner continues to maintain rejections based on the gene amplification assay (see pages 3-6 of the final Office Action). Since Appellants need show at least one utility to meet the requirements of 35 U.S.C. §101, instead of the gene amplification assay, the instantly pending claims were amended to rely upon assay 94 or 'the glucose/FFA uptake assay,' (Example 158) for patentable utility of PRO1182 polypeptides, in the responses filed March 10, 2005 and June 23, 2005. Accordingly, any of the Examiner's rejections /references or discussions referring to the gene amplification assay are not currently addressed. Only rejections directed to the glucose/FFA uptake utility are discussed below.

Patentable utility of the claimed PRO1182 polypeptides is based upon the results of the adipocyte glucose/FFA uptake assay for this polypeptide. The specification discloses that the

adipocyte glucose/FFA uptake assay is designed to determine whether a polypeptide is capable of modulating, either positively or negatively, the uptake of glucose or free fatty acids in adipocyte cells. By making such determinations, the assay identifies polypeptides that are expected to be useful for treating disorders wherein stimulation or inhibition of glucose uptake by adipocytes is expected to be therapeutically effective, for example, diabetes, and hyper- or hypo-insulinemia.

The glucose/FFA uptake assay, as described in Example 158 of the instant application, was well known in the art at the time of the effective filing date of the instant application. As demonstrated by the references of record, similar assays were commonly used to identify potential anti-diabetic agents. For instance, at the time of the effective filing date of the instant application, it was well known in the art that increasing glucose uptake by adipocyte cells is a hallmark of a number of therapeutically effective agents, such as troglitazone and pioglitazone. Treatment with vanadium salts, another agent which increased glucose uptake, was shown to lower glucose levels in hyperglycemic rats. Diabetes, hyperglycemia, and obesity were known at the time of filing to be closely linked conditions (see, for example, Sandouk, page 352). Thus, the art has shown that agents which decrease circulating FFA levels (or increase glucose/FFA uptake by adipocytes) are useful in the treatment of disorders such as diabetes, hyperglycemia, and obesity.

Appellants note that the evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence under consideration. Thus, to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner has made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the applicant. Therefore, the legal standard for patentable utility is not absolute certainty. Clear evidence supports the glucose/FFA uptake enhancing activity of PRO1182. On the other hand, the Examiner has provided no evidence to demonstrate that it more likely than not that one of skill in the art would doubt the truth of this asserted utility of PRO1182 as an enhancer of glucose/FFA uptake.

It is known in the art that FFA levels regulate glucose uptake by adipocytes. Thus, even if the actual mechanistic effect of PRO1182 is only to directly increase FFA uptake by adipocyte

cells, this will necessarily result in indirectly increasing glucose uptake by adipocytes. Furthermore, agents which are well known in the art as useful in the treatment of diabetes, such as the thiazolidenediones, have been shown to exert their effects, at least in part, through the increase of FFA uptake by adipocytes. Accordingly, an agent which increases FFA uptake by adipocytes has the same utility in the treatment of disease as those recognized by the Examiner for agents which enhance glucose uptake.

Accordingly, Appellants submit that when the proper legal standard is applied, one should reach the conclusion that the present application discloses at least one patentable utility for the claimed PRO1182 polypeptides. Further, based on this utility, one of skill in the art would know exactly how to use the claimed polypeptides without any undue experimentation.

Issue II: Written Description

Appellants note that the Advisory Action of November 1, 2006 does not repeat the rejection of Claims 122-123 under 35 U.S.C. §112, first paragraph, written description. The Examiner also failed to indicate if the rejection was withdrawn in the Advisory Action. Appellants argue this rejection as applied to pending Claims 122-123 assuming that the rejection has been maintained.

According to the Examiner, the subject matter was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors had possession of the claimed invention at the time of filing.

Amended Claims 122-123 claim the genus of polypeptides with at least 95-99% sequence identity to SEQ ID NO:357 with the functional recitation: “wherein said polypeptide enhances the uptake of glucose or FFA (free fatty acids) by adipocyte cells,” which is based on a well-established assay known to the skilled artisan at the effective filing date of this application. Moreover, the instant invention evidences the actual reduction to practice of full-length PRO1182 of SEQ ID NO: 357, with or without its signal sequence, or encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 203088. Therefore, the claimed polypeptides of Claims 122 and 123 are defined both by functional as well as structural features. Example 158, (page 530) of the present application provides detailed protocols for the glucose or FFA (free fatty acids) uptake assay by adipocyte cells including the extensive step-by-

step guidance in the specification. Appellants claim only those proteins which meet both recitations of the claims, structural and functional. Based on the detailed description of the cloning and expression of variants of PRO1182 in the specification, the description of the glucose or FFA (free fatty acids) uptake assay, the description of testing for variant polypeptides in the assay, the actual reduction to practice of sequence SEQ ID NO:357 and the functional recitation in the instant claims, one of skilled in the art would know that Appellants possessed the invention as claimed in the instant claims, at the time of filing of the application. From the specific activity of the claimed polypeptide, the description of the claimed genus is achieved.

Accordingly, Appellants respectfully request reconsideration and withdrawal of this outstanding rejection under 35 U.S.C. §112, first paragraph.

These arguments are all discussed in further detail below under the appropriate headings.

ISSUE I: Claims 122-126, 129-131 satisfy the utility requirement of 35 U.S.C. §101 and 35 U.S.C. §112, first paragraph

Claims 122-126, 129-131 stand rejected under 35 U.S.C. §101 because allegedly “the claimed invention is not supported by either a specific and substantial asserted utility or a well-established utility.” (Page 2 of the final Office Action mailed April 12, 2006).

Appellants submit, for the reasons set forth below, that the specification discloses at least one credible, substantial and specific asserted utility for the PRO1182 polypeptides.

A. The Legal Standard for Utility

According to 35 U.S.C. § 101:

Whoever invents or discovers any new and *useful* process, machine, manufacture, or composition of matter, or any new and *useful* improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title. (Emphasis added).

In interpreting the utility requirement, in *Brenner v. Manson*,¹ the Supreme Court held that the *quid pro quo* contemplated by the U.S. Constitution between the public interest and the interest of the inventors required that a patent applicant disclose a “substantial utility” for his or

¹ *Brenner v. Manson*, 383 U.S. 519, 148 U.S.P.Q. (BNA) 689 (1966).

her invention, i.e. a utility “where specific benefit exists in currently available form.”² The Court concluded that “a patent is not a hunting license. It is not a reward for the search, but compensation for its successful conclusion. A patent system must be related to the world of commerce rather than the realm of philosophy.”³

Later, in *Nelson v. Bowler*,⁴ the C.C.P.A. acknowledged that tests evidencing pharmacological activity of a compound may establish practical utility, even though they may not establish a specific therapeutic use. The court held that “since it is crucial to provide researchers with an incentive to disclose pharmaceutical activities in as many compounds as possible, we conclude adequate proof of any such activity constitutes a showing of practical utility.”⁵

In *Cross v. Iizuka*,⁶ the C.A.F.C. reaffirmed *Nelson*, and added that *in vitro* results might be sufficient to support practical utility, explaining that “*in vitro* testing, in general, is relatively less complex, less time consuming, and less expensive than *in vivo* testing. Moreover, *in vitro* results with the particular pharmacological activity are generally predictive of *in vivo* test results, i.e. there is a reasonable correlation there between.”⁷ The court perceived “No insurmountable difficulty” in finding that, under appropriate circumstances, “*in vitro* testing, may establish a practical utility.”⁸

The case law has also clearly established that Appellants’ statements of utility are usually sufficient, unless such statement of utility is unbelievable on its face.⁹ The PTO has the initial burden to prove that Appellants’ claims of usefulness are not believable on their face.¹⁰ In

² *Id.* at 534, 148 U.S.P.Q. (BNA) at 695.

³ *Id.* at 536, 148 U.S.P.Q. (BNA) at 696.

⁴ *Nelson v. Bowler*, 626 F.2d 853, 206 U.S.P.Q. (BNA) 881 (C.C.P.A. 1980).

⁵ *Id.* at 856, 206 U.S.P.Q. (BNA) at 883.

⁶ *Cross v. Iizuka*, 753 F.2d 1047, 224 U.S.P.Q. (BNA) 739 (Fed. Cir. 1985).

⁷ *Id.* at 1050, 224 U.S.P.Q. (BNA) at 747.

⁸ *Id.*

⁹ *In re Gazave*, 379 F.2d 973, 154 U.S.P.Q. (BNA) 92 (C.C.P.A. 1967).

¹⁰ *Ibid.*

general, an Applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. §101, "unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope."^{11,12}

Compliance with 35 U.S.C. §101 is a question of fact.¹³ The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence under consideration.¹⁴ Thus, to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the applicant. The issue will then be decided on the totality of evidence.

The well established case law is clearly reflected in the Utility Examination Guidelines ("Utility Guidelines")¹⁵, which acknowledge that an invention complies with the utility requirement of 35 U.S.C. §101, if it has at least one asserted "specific, substantial, and credible utility" or a "well-established utility." Under the Utility Guidelines, a utility is "specific" when it is particular to the subject matter claimed. For example, it is generally not enough to state that a nucleic acid is useful as a diagnostic without also identifying the conditions that are to be diagnosed.

In explaining the "substantial utility" standard, M.P.E.P. §2107.01 cautions, however, that Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations used in certain court decisions to mean that products or services based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. "Rather, any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with

¹¹ *In re Langer*, 503 F.2d 1380,1391, 183 U.S.P.Q. (BNA) 288, 297 (C.C.P.A. 1974).

¹² *See also In re Jolles*, 628 F.2d 1322, 206 U.S.P.Q. 885 (C.C.P.A. 1980); *In re Irons*, 340 F.2d 974, 144 U.S.P.Q. 351 (1965); *In re Sichert*, 566 F.2d 1154, 1159, 196 U.S.P.Q. 209, 212-13 (C.C.P.A. 1977).

¹³ *Raytheon v. Roper*, 724 F.2d 951, 956, 220 U.S.P.Q. (BNA) 592, 596 (Fed. Cir. 1983) *cert. denied*, 469 US 835 (1984).

¹⁴ *In re Oetiker*, 977 F.2d 1443, 1445, 24 U.S.P.Q.2d (BNA) 1443, 1444 (Fed. Cir. 1992).

¹⁵ 66 Fed. Reg. 1092 (2001).

regard to defining a ‘substantial’ utility.”¹⁶ Indeed, the Guidelines for Examination of Applications for Compliance With the Utility Requirement,¹⁷ gives the following instruction to patent examiners: “If the applicant has asserted that the claimed invention is useful for any particular practical purpose . . . and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility.”

B. The results of the adipocyte glucose/FFA uptake assay provide utility for the PRO1182 polypeptide

The Examiner continues to maintain rejections based on the gene amplification assay (see pages 3-6 of the final Office Action). Since Appellants need show at least one credible, substantial and specific asserted utility for the PRO1182 polypeptides to meet the requirements of 35 U.S.C. §101, as indicated instead of the gene amplification assay, the instantly pending claims were amended to rely upon assay 94 or ‘the glucose/FFA uptake assay,’ (Example 158) for patentable utility of PRO1182 polypeptides, in the responses filed March 10, 2005 and June 23, 2005. Accordingly, any of the Examiner’s rejections /references or discussions referring to the gene amplification assay are not currently addressed. Only rejections directed to the glucose/FFA uptake utility are discussed below.

Appellants respectfully submit that they rely on the adipocyte glucose/FFA uptake assay) for patentable utility of the claimed PRO1182 polypeptides, and that the adipocyte glucose/FFA uptake assay data for the PRO1182 polypeptide is clearly disclosed in the instant specification under Example 158.

The adipocyte glucose/FFA uptake assay is designed to determine whether a polypeptide is capable of modulating, either positively or negatively, the uptake of glucose or free fatty acids in adipocyte cells. By making such determinations, the assay identifies polypeptides that are expected to be useful for treating disorders wherein stimulation or inhibition of glucose uptake by adipocytes is expected to be therapeutically effective. Examples of these types of disorders include obesity, diabetes, and hyper- or hypo-insulinemia.

¹⁶ M.P.E.P. §2107.01.

¹⁷ M.P.E.P. §2107 II(B)(1).

The adipocyte glucose/FFA assay is performed as follows: primary rat adipocyte cells are plated on a 96 well plate and incubated overnight with media supplemented with PRO1182 polypeptide. After the initial overnight incubation, samples of the media are taken at hour 4 and hour 16 and residual glycerol, glucose and FFA are measured. After the hour 16 sample is taken, insulin is added to the media and the adipocytes are allowed to incubate for an additional 4 hours. After this final 4 hour incubation, another sample is taken and residual glycerol, glucose and FFA is measured again. As a control, identical incubations and samplings are performed on cells that have been incubated overnight in media initially supplemented with insulin rather than PRO1182 polypeptide. Results are scored as positive in the assay if the uptake is greater than 1.5 times (stimulatory) or less than 0.5 times (inhibitory) the uptake of the insulin control. As PRO1182 resulted in more than 1.5 times the uptake of the insulin control, PRO1182 tested positive as a stimulator of glucose/FFA uptake in adipocyte cells.

The glucose/FFA uptake assay as described in Example 158 of the instant application was also well known in the art at the time of the effective filing date of the instant application. Similar assays were commonly used to identify potential anti-diabetic agents and to study the regulatory mechanisms of important molecules involved in fat cell metabolism.

For example, at the time of the effective filing date of the instant application, it was well known in the art that increasing glucose uptake by adipocyte cells is a hallmark of a number of therapeutically effective agents, such as troglitazone and pioglitazone. (Tafari, *Endocrinology*, 137(11): 4706-4712 (1996); Sandouk, *et al.*, *Endocrinology*, 133(1):352-359 (1993)). Both troglitazone and pioglitazone are members of the thiazolidinedione class of compounds and have been used to effectively treat noninsulin-dependent diabetes mellitus (NIDDM), the most common form of diabetes. Both compounds function, at least in part, by increasing the number of cellular glucose transporters in order to facilitate increased glucose uptake.

Further, at the time of the effective filing date of the instant application, vanadium salts were considered as a possible treatment for diabetes, and several clinical trials had already been performed. (Page 26617, right column, Goldwasser *et al.*, *J. Biol Chem.*, 274(37):26617-26624 (1999)). Using a rat adipocyte culture system similar to the system disclosed in the instant application, Goldwasser *et al.*, showed that vanadium ligand 1-Glu (γ)HXM potentiates the capacity of free vanadium ions to activate glucose uptake and glucose metabolism in rat adipocytes *in vitro* by 4-5 folds and to lower blood glucose levels in hyperglycemic rats *in vivo*

by 5-7 fold. This is further evidence that at the effective filing date of the present application one skilled in the art would have reasonably expected that molecules activating glucose uptake would find utility in the treatment of diabetes and related diseases.

In addition, the investigators in Mueller *et al.*, who were interested in determining the influence of glucose uptake on leptin secretion, employed essentially the same assay to measure changes in glucose uptake after insulin exposure. (Mueller *et al.*, *Endocrinology*, 139(2): 551-558 (1998)). Figure 1A shows the glucose concentrations in medium from 0-96 hours from isolated rat adipocytes in primary culture with various insulin concentrations. As indicated by the decrease in glucose in the medium in the Figure, Mueller *et al.* suggest that insulin produced a concentration-dependent increase in glucose uptake by the cultured adipocytes. Based on these experimental results, the authors stated that insulin increased leptin secretion over 96 hours, and that the increase in leptin was closely related to the amount of glucose taken up by the adipocytes than to the insulin concentration, suggesting a role for glucose transport and/or metabolism in regulating leptin secretion. (See Abstract).

Using the same assay system, Mueller *et al.* further studied the effect on leptin secretion of two well-known anti-diabetic agents, metformin and vanadium, which were known to enhance glucose uptake. (Muller *et al.*, *Obesity Research*, 8(7): 530-539 (2000)). The experimental data indicated that both metformin and vanadium increased glucose uptake and inhibit leptin secretion from cultured adipocytes.

Accordingly, Appellants respectfully submit that at the effective filing date of the instant application, one of skill in the art would have reasonably accepted that various compounds, such as PRO1182, that are capable of modulating glucose uptake have a substantial, practical, real life utility. The above-mentioned studies have clearly established that the glucose/FFA uptake assay as described in the instant application is a reliable assay system to identify therapeutic agents for treating diseases and conditions such as obesity, diabetes, and hyperinsulinemia. Therefore, Appellants respectfully submit that a variety of real-life utilities, such as treatments for glucose uptake related diseases, including obesity and diabetes, are envisioned for PRO1182 based on the glucose/FFA uptake assay results disclosed herein.

The Examiner has acknowledged that the teachings within "each of the references cited by the Applicant (Tafari *et al.*, Sandouk *et al.*, Goldwasser *et al.*, Mueller *et al.* (1998) and Mueller *et al.* (2000)) teaches that the agents utilized in the assays enhance glucose

uptake.....Disorders such as obesity, diabetes, and hyper- or hypo-insulinemia are characterized by a reduction in the amount of glucose entering all cells, including adipocytes.....Therefore, as emphasized by Tafuri *et al.*, Sandouk *et al.*, Goldwaser *et al.*, Mueller *et al.* (1998) and Mueller *et al.* (2000), one skilled in the art is searching for agents that will enhance glucose uptake into adipocyte cells.” (page 7, line 2 through page 8, line 2 of final Office Action of April 12, 2006).

As the Examiner acknowledges, based on the instant results demonstrating the ability of the PRO1182 polypeptides to enhance glucose uptake in the glucose/ FFA assay, one skilled in the art would readily recognize that PRO1182 polypeptides are useful in the treatment of disorders benefiting from this biological activity, such as obesity, diabetes, or hyper- or hypo-insulinemia.

The Examiner has further maintained that “Tafuri *et al.*, Sandouk *et al.*, Goldwaser *et al.*, Mueller *et al.* (1998) and Mueller *et al.* (2000) teach different methodologies for the measurement of glucose uptake in adipocyte cells as compared to the glucose assay of the instant specification....None of the references utilizes the same grading scale disclosed in the instant specification, but instead report dose-response curves. The instant specification does not report any specific cell numbers or statistical differences and there is no indication in the specification as to how PRO1182 inhibited glucose uptake as compared to control or whether the results were significant” (Emphasis added). The Examiner concludes that the PRO1182 peptide is not in currently available form, and the asserted utility is not substantial.

Appellants respectfully submit that, compliance with the utility requirement does not require that the methodology used in making the invention be the same as those used in the referenced or related art. What is important is that the assay be a well- recognized assay and that guidelines be provided in the specification to perform the assay, including assay read-out, if applicable. As discussed in their response dated August 10, 2006, Appellants submitted that the glucose uptake assay is a well-accepted assay in the art for identifying molecules that modulate glucose uptake. The fact remains that the results of the adipocyte glucose/FFA uptake assay were positive, indicating that PRO1182 polypeptides are useful in enhancing glucose uptake by adipocyte cells. The instant specification also clearly discusses the controls used in the assay. For example, the results of the glucose uptake assay were scored as positive if the uptake was greater than 1.5 times (stimulatory), or as inhibitory, if the uptake was less than 0.5 times the

uptake of the insulin control. Since PRO1182 resulted in more than 1.5 times the uptake of the insulin control, PRO1182 tested positive as a **stimulator** (or enhancer) of glucose/FFA uptake in adipocyte cells.

The Examiner's requirement for specific "same grading scales", "specific cell numbers or statistical differences" (page 9, paragraph 1 of final Office Action) are also clearly not a requirement of the utility standards set by the USPTO. Appellants submit that the glucose uptake assay described herein is a comparative assay, meaning that the utility is based upon a comparison of relative uptake levels between a well-accepted and known control like insulin (for glucose uptake) and a test molecule like PRO1182. Useful pharmacological information is obtained when a relative difference is observed in this assay. In addition, the need for "cell numbers or statistical results" is a misplaced requirement, and is a clear indication that the Examiner applies a standard that might be appropriate if the issue at hand were the regulatory approval of a pharmacological or diagnostic assay, but is fully inappropriate for determining if the "utility" standard of the Patent Statute is met. The FDA, reviewing an application for a new assay, will indeed ask for actual numerical data, statistical analysis, and other specific information before any assay is approved. However, the Patent and Trademark Office is not the FDA, and the standards of patentability are not the same as the standards of market approval. It is well established law that therapeutic utility sufficient under the patent laws is not to be confused with the requirements of the FDA with regard to safety and efficacy of drugs to marketed in the United States.

C. A prima facie case of lack of utility has not been established

The Examiner has asserted that "assays demonstrating that glucose uptake is inhibited by the PRO polypeptide are not enabling, since there is no real world use for such a peptide, or the assay that measures it. Since there is little additional information about the claimed PRO peptide, use of the claimed peptide for the FFA assay cannot be seen as enabling without evidence or data supporting a specific function for the claimed PRO amino acids" (Page 2 of the Advisory Action mailed November 1, 2006).

Appellants respectfully point out that the standard for utility is not absolute certainty, but more likely than not. Appellants further point out that it was well known in the art at the time of

filing that both glucose and FFA levels were associated with diabetes, obesity, and hyperinsulinemia. In fact, it was further known in the art at the time of filing that, antidiabetic agents such as the thiazolidinediones (including troglitazone and pioglitazone) which increase glucose uptake, also increase FFA uptake by adipocytes (see references (Tafari, *Endocrinology*, 137(11): 4706-4712 (1996); Sandouk, *et al.*, *Endocrinology*, 133(1):352-359 (1993) submitted by Appellants). Thus, circulating FFA level was one of the factors which regulated glucose uptake. Hence, it was clear that agents which decreased circulating FFA levels were effective in the treatment of diseases such as obesity and diabetes. In other words, agents which increased FFA uptake by adipocytes were useful in the treatment of diabetes, obesity and hyperinsulinemia.

Thus, even if the actual mechanistic effect of PRO1182 is only to directly increase FFA uptake by adipocyte cells, this will necessarily result in indirectly increasing glucose uptake by adipocytes. The effect of FFA levels on glucose uptake has been clearly demonstrated in the references in the art at the time of filing of the application. Furthermore, agents which are well known in the art as useful in the treatment of diabetes, such as the thiazolidinediones, have been shown to exert their effects, at least in part, through the increase of FFA uptake by adipocytes. Accordingly, an agent which increases FFA uptake by adipocytes has the same utility in the treatment of disease as those recognized by the Examiner for agents which increase glucose uptake.

Accordingly, one of ordinary skill in the art would find it more likely than not that an agent which increases uptake of glucose and/or FFA by adipocytes would also be useful in the treatment of disorders such as diabetes, hyperglycemia, and obesity. Clear evidence supports the glucose/FFA uptake enhancing activity of PRO1182. On the other hand, the Examiner has provided no evidence to demonstrate that it more likely than not that one of skill in the art would doubt the truth of this asserted utility of PRO1182 as an enhancer of glucose/FFA uptake. Since the standard is not absolute certainty, a *prima facie* showing of lack of utility has not been made in this instance and the burden to provide further evidence of utility has not shifted to Appellants.

Based on the above arguments, Appellants have clearly demonstrated a credible, specific and substantial asserted utility for the PRO1182 polypeptide, for example in the treatment of disorders such as obesity, diabetes, and hyper- or hypo-insulinemia. Further, based on this utility and the disclosure in the specification, one skilled in the art at the time the application was filed

would know how to use the PRO1182 polypeptides, for instance, in the treatment of disorders for which modulation of glucose uptake by adipocytes would be beneficial, such as obesity, diabetes, and hyper- or hypo-insulinemia without undue experimentation.

Accordingly, given that the specification discloses at least one patentable utility for the claimed PRO1182 polypeptides, Appellants respectfully request reconsideration and reversal of the rejection of Claims 122-126, 129-131 under 35 U.S.C. §101 and 35 U.S.C. §112, first paragraph.

ISSUE II: Claims 122-123 satisfy the written description requirement of 35 U.S.C. §112, first paragraph.

Claims 122-123 stand rejected under 35 U.S.C. §112, first paragraph, allegedly “because, the subject matter was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors had possession of the claimed invention at the time of filing.” (Page 11 of the final Office Action mailed April 12, 2006).

Appellants note that the Advisory Action of November 1, 2006 does not repeat the rejection of Claims 122-123 under 35 U.S.C. §112, first paragraph, written description. The Examiner also failed to indicate if the rejection was withdrawn in the Advisory Action. Appellants argue this rejection as applied to pending Claims 122-123, assuming that the rejection has been maintained.

Appellants were in possession of the invention as of the effective filing date of an application is a factual determination, reached by the consideration of a number of factors, including the level of knowledge and skill in the art, and the teaching provided by the specification. The inventor is not required to describe every single detail of his/her invention. An Applicant's disclosure obligation varies according to the art to which the invention pertains.

The currently amended claims claim the genus of polypeptides with at least 95-99% sequence identity to SEQ ID NO:357 with the functional recitation: “wherein said polypeptide enhances the uptake of glucose or FFA (free fatty acids) by adipocyte cells,” which, as discussed above, is based on a well-established assay known to the skilled artisan at the effective filing date of this application. Moreover, the instant invention evidences the actual reduction to practice of full-length PRO1182 of SEQ ID NO: 357, with or without its signal sequence, or encoded by the full-length coding sequence of the cDNA deposited under ATCC accession

number 203088. Therefore, the claimed polypeptides of Claims 122 and 123 are defined both by functional as well as structural features.

The present situation is analogous to Example 14 on pages 53-55 of the Written Description Training Materials which analyzes a claim directed to a protein and variants thereof having 95% sequence identity, all of which share the same biological function, for its compliance with the written description requirement of 35 U.S.C. §112, first paragraph. The Written Description Training Materials conclude that such a claim satisfies the written description requirement of 35 U.S.C. §112, first paragraph, when: (1) a single protein sequence is actually reduced to practice, (2) procedures for making variants of that "reduced to practice" protein sequence are conventional in the art, and (3) an assay is described which allows identification of other proteins having the same biological activity. The reasoning provided by the USPTO in the Written Description Training Materials is that:

“[t]here is actual reduction to practice of the single disclosed species. The specification indicates that the *genus of proteins that must be variants of SEQ ID NO:...* does not have substantial variation since all of the variants must possess the specified [biological function] and must have at least 95% identity to the reference sequence, SEQ ID NO:.... The single species disclosed *is representative of the genus* because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO:.... which are capable of the specified [biological function]. One of skill in the art would conclude that applicant was in possession of the necessary common attributes possessed by members of the genus..... {As such}, the disclosure meets the requirements of 35 U.S.C. § 112, first paragraph, as providing adequate written description for the claimed invention.” (emphasis added).

The instantly pending claims recite polypeptides having 95% or 99% sequence identity with the disclosed polypeptide sequence SEQ ID NO: 357, and also include the functional recitation: “wherein said polypeptide enhances the uptake of glucose or FFA (free fatty acids) by adipocyte cells.” Example 158, (page 530) of the present application provides detailed protocols for the glucose or FFA (free fatty acids) uptake assay by adipocyte cells including the extensive step-by-step guidance in the specification. Appellants claim only those proteins which meet both recitations of the claims, structural and functional. The specification further describes methods for the determination of percent identity between two amino acid sequences. In fact, the specification teaches specific parameters to be associated with the term “percent identity” as applied to the present invention (page 306-308, line 14 onwards). The specification further

provides detailed guidance as to changes that may be made to a PRO polypeptide without adversely affecting its activity (page 372, line 36 to page 373, line 17). This guidance includes a listing of exemplary and preferred substitutions for each of the twenty naturally occurring amino acids (Table 6, page 372). Accordingly, one of skill in the art could identify whether a variant PRO1182 sequence falls within the parameters of the claimed invention. Once such an amino acid sequence is identified, the specification sets forth methods for making the amino acid sequences (see page 305, line 23 and page 371) and methods of preparing the PRO polypeptides (Example 140-143, page 376).

Based on the detailed description of the cloning and expression of variants of PRO1182 in the specification, the description of the glucose or FFA (free fatty acids) uptake assay, the description of testing for variant polypeptides in the assay, the actual reduction to practice of sequence SEQ ID NO:357 and the functional recitation in the instant claims, one of skilled in the art would know that Appellants possessed the invention as claimed in the instant claims, at the time of filing of the application. From the specific activity of the claimed polypeptide, Appellants believe that the description of the claimed genus is achieved.

Accordingly, Appellants respectfully request reconsideration and reversal of the written description rejection of Claims 122-123 under 35 U.S.C. §112, first paragraph.


CONCLUSION

For the reasons given above, Appellants submit that the adipocyte glucose/FFA uptake assay disclosed in Example 158 of the specification provides at least one asserted specific and substantial patentable utility for the claimed PRO1182 polypeptides, and that one of ordinary skill in the art would accept this asserted utility as credible, and would understand how to make and use the claimed PRO1182 polypeptides. Therefore, Claims 122-126, 129-131 meet the requirements of 35 U.S.C. §101 and 35 U.S.C. §112, first paragraph, for Utility and Enablement. Further, for the reasons outlined above, the requirements of 35 U.S.C. §112, first paragraph, Written Description are also met. Accordingly, reversal of all the rejections of 122-126, 129-131 is respectfully requested.

Please charge any additional fees, including fees for additional extension of time, or credit overpayment to Deposit Account No. 08-1641 (referencing Attorney's Docket No. 39780-2730 P1C33).

Respectfully submitted,

Date: May 2, 2007

By: 
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8. CLAIMS APPENDIX

Claims on Appeal

122. An isolated polypeptide having at least 95% amino acid sequence identity to:
- (a) the amino acid sequence of the polypeptide of SEQ ID NO:357;
 - (b) the amino acid sequence of the polypeptide of SEQ ID NO:357, lacking its associated signal peptide; or
 - (c) the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 203088;
- wherein said polypeptide enhances the uptake of glucose or FFA (free fatty acids) by adipocyte cells.
123. An isolated polypeptide having at least 99% amino acid sequence identity to:
- (a) the amino acid sequence of the polypeptide of SEQ ID NO:357;
 - (b) the amino acid sequence of the polypeptide of SEQ ID NO:357, lacking its associated signal peptide; or
 - (c) the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 203088;
- wherein said polypeptide enhances the uptake of glucose or FFA (free fatty acids) by adipocyte cells.
124. An isolated polypeptide comprising:
- (a) the amino acid sequence of the polypeptide of SEQ ID NO:357;
 - (b) the amino acid sequence of the polypeptide of SEQ ID NO:357, lacking its associated signal peptide; or
 - (c) the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 203088.

125. The isolated polypeptide of Claim 124 comprising the amino acid sequence of the polypeptide of SEQ ID NO:357.
126. The isolated polypeptide of Claim 124 comprising the amino acid sequence of the polypeptide of SEQ ID NO:357, lacking its associated signal peptide.
129. The isolated polypeptide of Claim 124 comprising the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 203088.
130. A chimeric polypeptide comprising a polypeptide according to Claim 124 fused to a heterologous polypeptide.
131. The chimeric polypeptide of Claim 130, wherein said heterologous polypeptide is an epitope tag or an Fc region of an immunoglobulin.

9. EVIDENCE APPENDIX

1. Tafuri, S.R., "Troglitazone Enhances Differentiation, Basal Glucose Uptake, and Glut1 Protein Levels in 3T3-L1 Adipocytes," *Endocrinology*, 137(11):(4706-4712 (1996).
2. Sandouk, T., et al., "The Antidiabetic Agent Pioglitazone Increases Expression of Glucose Transporters in 3T3-F442A Cells by Increasing Messenger Ribonucleic Acid Transcript Stability", *Endocrinology* 133(1):352-359 (1993).
3. Goldwasser, I., et al., "L-Glutamic Acid γ -Monohydroxamate: A potentiator of vanadium-evoked glucose metabolism *in vitro* and *in vivo*", *J. Biol. Chem.* 274(37):26617-26624 (1999).
4. Mueller, W. M., et al., "Evidence That Glucose Metabolism Regulates Leptin Secretion from Cultured Rat Adipocytes", *Endocrinology* 139(2):551-558 (1998).
5. Mueller, W. M., et al., "Effects of Metformin and Vanadium on Leptin Secretion from Cultured Rat Adipocytes", *Obesity Research* 8(7):530-539 (2000).

Items 1-5 were submitted with Appellants' Information disclosure Statement filed March 10, 2005, and was made of record by the Examiner in the Office Action mailed September 26, 2005.

10. RELATED PROCEEDINGS APPENDIX

None- - no decision rendered by a Court or the Board in any related proceedings identified above.

Troglitazone Enhances Differentiation, Basal Glucose Uptake, and Glut1 Protein Levels in 3T3-L1 Adipocytes

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ABSTRACT

Troglitazone is a member of the thiazolidinedione class of compounds, which act as insulin-sensitizing agents when administered to human patients and animal models displaying noninsulin-dependent diabetes mellitus. In Zucker rats, the antidiabetic activity is associated with increased glucose uptake in adipose tissue. To understand the direct effects troglitazone has on adipocyte metabolism, 3T3-L1 preadipocytes and adipocytes were treated with the compound. The addition of troglitazone enhanced the rate and percent differentiation of fibroblasts to adipocytes. Northern analysis indicated that during differentiation, expression of the adipocyte-specific transcription factor, CCAAT enhancer binding protein- α , increased more rapidly in

troglitazone-treated cells, but did not change in fully differentiated adipocytes. To assess the metabolic consequences of troglitazone treatment, both basal and insulin-stimulated glucose uptake were monitored in treated cells. Troglitazone treatment increased basal glucose transport 1.5- to 2.0-fold, whereas insulin-stimulated uptake was unaffected. Enhanced basal transport was caused by an increased synthesis of both Glut1 glucose transporter messenger RNA and protein. These results suggest the possibility that *in vivo*, the troglitazone-dependent increase in glucose disposal may be attributable in part to modifications in the expression of Glut1 in insulin-responsive tissues. (*Endocrinology* 137: 4706-4712, 1996)

NONINSULIN-DEPENDENT diabetes mellitus (NIDDM), the most common form of diabetes, is estimated to affect more than 4 million people in the United States (1). This disease commonly develops during middle age and is characterized by hyperglycemia, hyperinsulinemia, and insulin resistance. At present, treatment consists of behavioral modifications in conjunction with the administration of insulin and oral hypoglycemic agents (sulfonylureas and biguanide compounds). However, these treatments often fail to ameliorate one of the main underlying causes of the disease, insulin resistance. The thiazolidinediones, a new class of compounds, differ markedly from other antidiabetic agents in that they decrease hyperglycemia and hyperinsulinemia by increasing insulin sensitivity in target tissues. These compounds increase peripheral glucose uptake while decreasing insulin secretion and gluconeogenesis in a wide variety of type II animal models (2-4). Troglitazone, a member of this compound class, improves glucose tolerance and insulin sensitivity in both diabetic (5, 6) and glucose-intolerant (7) patients. Little is known about the biochemical mechanism of action of these compounds.

Adipose tissue is highly responsive to insulin. Its primary role is to store energy when nutrients are plentiful and to release energy during fasting and starvation. Adipose tissue also plays a pivotal role in metabolic homeostasis. Adipose tissue is responsible for 50-70% of lactate production in the adult and consequently contributes to the regulation of glycogen synthesis and gluconeogenesis (8). Moreover, the recent discovery of the ob gene product indicates that adipose

tissue secretes hormones capable of regulating feeding patterns, satiety, and adiposity (9). Because of the importance of adipose tissue in metabolic regulation and insulin resistance, it may play an important role in the mechanism of action of thiazolidinediones.

To understand if and how the thiazolidinediones affect adipose cell metabolism, a study was designed to determine how troglitazone affects glucose utilization in 3T3-L1 adipocytes. This system was chosen because it is easily manipulated and is not complicated by the problems associated with the metabolic feedback loops present *in vivo*. Additionally, previous experiments with pioglitazone, another member of the thiazolidinedione family, in 3T3-F442A adipose cells suggested that these compounds increase differentiation (10, 11) and glucose uptake (12) in adipocytes. In the studies discussed here, troglitazone increased differentiation in 3T3-L1 cells when administered at the initiation of the differentiation protocol. Furthermore, troglitazone enhanced glucose uptake in these cells by altering the total number of basal glucose transporters within the cell.

Materials and Methods

Materials

3T3-L1 cells were obtained from the American Type Tissue Culture Collection (Rockville, MD). High glucose DMEM culture medium and bovine serum were purchased from Life Technologies (Gaithersburg, MD). Insulin, dexamethasone, and isomethylbutylxanthine were purchased from Sigma Chemical Co. (St. Louis, MO). Troglitazone and pioglitazone were synthesized by Parke-Davis (Ann Arbor, MI) and Sankyo (Tokyo, Japan), respectively.

Cell culture

3T3-L1 fibroblasts were grown and passaged in DMEM containing 10% FBS. For adipocyte differentiation, 2 day postconfluent cells were placed in 10% FBS-DMEM, 1 μ g/ml insulin, 0.25 μ M dexamethasone,

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0.5 mM isomethylbutylxanthine. Forty-eight hours later, the medium was changed to 10% FBS-DMEM containing 1 μ g/ml insulin, and after an additional 48 h, the medium was replaced with 10% FBS-DMEM. Thereafter, the medium was changed every 2 days. Troglitazone was dissolved in dimethylsulfoxide as a 1000-fold stock solution and administered at the initiation of differentiation and with every medium change unless otherwise indicated.

Glucose uptake

Basal glucose uptake was measured using a modified version of the glucose transport procedure described by de Herreros and Birnbaum [3]. Briefly, cells were washed with PBS and placed in glucose-free MEM containing 1% BSA for 30 min at 37°C. At this time, 1 μ Ci/ml [3 H]-2-deoxyglucose and 1 mM glucose were added, and the cells were incubated for 30 min at 22°C. Subsequently, the cells were washed with PBS and 10 mM glucose at 4°C and lysed with 0.5 N NaOH. The lysates were neutralized with acetic acid and counted. To measure insulin-stimulated glucose uptake, cells were serum starved for 3 h before initiation of the assay. Stimulation with insulin (1×10^{-6} M) was initiated 5 min before the addition of labeled glucose.

RNA preparation, Northern blot analysis, and ribonuclease RNase) protection assays

The guanidinium lysis method (Ultraspec RNA isolation system, Biotecx, Houston, TX) was used to prepare RNA samples. For Northern analysis, 15–30 μ g RNA/sample were resolved on an 0.8–1% agarose formaldehyde gel and transferred to nitrocellulose. The blots were hybridized in 1 M NaCl, 1% SDS, and 10% dextran sulfate for 16–20 h at 2°C. Washes were conducted in $0.1 \times$ SSC (standard saline citrate)-0.1% SDS at 50°C. The indicated complementary DNAs were labeled with α - 32 P]deoxy-CTP using the random prime method and used as probes. RNase protection assays were performed using the RNase A nuclease assay according to manufacturer's instructions (Ambion, Houston, TX). α - 32 P]UTP-labeled single stranded RNA probes were generated using T3/T7 *in vitro* transcription procedures. All results were quantitated using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Western blot analysis

Cell lysates were prepared in HNTG buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol, and 1 mM EDTA). The Bio-Rad protein analysis system (Bio-Rad Laboratories, Richmond, CA) was used to determine protein content. Western blots were performed using standard protocols. Antibody interactions were detected using the chemiluminescent assay (ECL, Amersham, Arlington Heights, IL). The Glut1 antibody was produced using a glutathione S-transferase fusion protein encoding the last 39 amino acids of the Glut1 glucose transporter. The antibody was immunoaffinity purified and shown not to cross-react with purified Glut4 glucose transporter protein. The Glut4-specific antibody was kindly provided by Dr. Michael Mueckler.

Results

Troglitazone enhances adipocyte differentiation in 3T3-L1 cells

The differentiation of 3T3-L1 preadipocytes into adipocytes is a complex process that is affected by cell passage number and a variety of environmental conditions. Under ideal conditions, 95–100% of confluent preadipocytes can be converted into fat droplet-containing adipocytes in 5–7 days, with fat droplets first appearing on day 4. However, as the preadipocytes are passaged, the efficiency of differentiation diminishes in a clonal manner, such that a confluent plate will contain islands of adipocytes within undifferentiated fibroblasts. This loss of phenotype is also associated with a decrease in the rate of differentiation, causing the initial appearance of fat droplets to occur around day 5 or 6. Initial experiments were conducted using cells with an adipocyte

conversion frequency of approximately 50%. To study the effects of troglitazone on differentiation, 0.5–5 μ M compound was added with 167 nM insulin, 0.25 μ M dexamethasone, and 0.5 mM isomethylbutylxanthine (hormone cocktail) at the initiation of differentiation and reapplied with each medium change. Eight-day troglitazone treatment without hormone cocktail showed minimal differentiation (Fig. 1, A and B). Less than 1% of the cells became adipocytes; however, the number of adipocytes in the drug-treated sample was greater than that in the untreated sample. In the presence of hormone cocktail, troglitazone significantly enhanced the percentage of adipocyte differentiation (Fig. 1, C and D). Nearly 100% of treated cells contained fat droplets compared to 50% of the untreated group. Additionally, the rate of differentiation was enhanced as fat droplets began to accumulate in the treated group 1 day before the control group.

Adipocyte differentiation has been shown to be dependent upon the activation of several transcription factors, which, in turn, initiate the expression of a repertoire of adipocyte genes. One such factor, CCAAT enhancer binding protein- α (C/EBP α), has been shown to be necessary and sufficient for adipocyte conversion in both preadipocyte and fibroblast cell lines [14–17]. Moreover, mice lacking C/EBP α expression fail to accumulate both white and brown fat, suggesting that C/EBP α is required for terminal adipocyte differentiation [18]. C/EBP α mRNA is not expressed in preadipocytes, is induced 2–3 days after the initiation of differentiation, and is maintained at a high level in the adipocyte [19]. As troglitazone increases adipocyte differentiation, total RNA was isolated from differentiating cells treated with hormone cocktail containing 0 or 5 μ M troglitazone and probed with labeled C/EBP α complementary DNA to determine whether troglitazone enhances C/EBP α expression. The control cells used in this experiment differentiated 95–100% without the

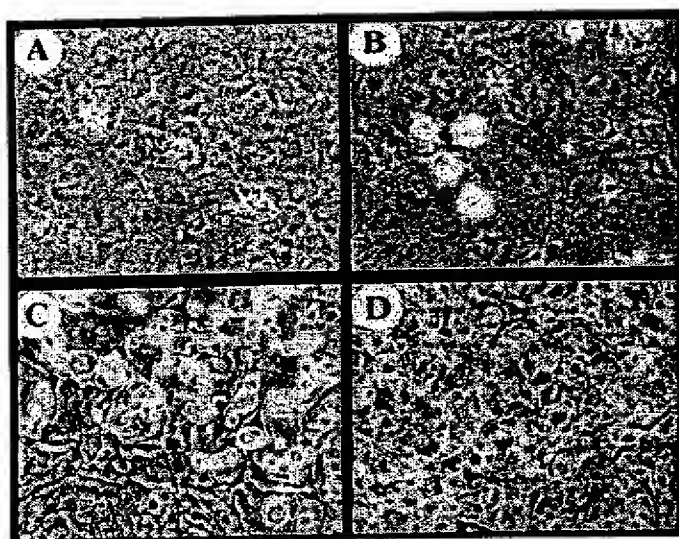


FIG. 1. Troglitazone enhances the differentiation of 3T3-L1 preadipocytes to adipocytes. Confluent preadipocytes were maintained in culture for 8 days without compound (A) or with 5 μ M troglitazone (B), as described in *Materials and Methods*. Identical cells were treated with differentiation cocktail (167 nM insulin, 0.25 μ M dexamethasone, and 0.5 mM isomethylbutylxanthine) with (D) or without (C) 5 μ M troglitazone.

lition of drug; therefore, the ratio of adipocytes/preadipocytes in the drug-treated and untreated samples was identical. As shown in Fig. 2, troglitazone increased the rate of EBP α mRNA accumulation during differentiation, but did not affect the levels of C/EBP α message after differentiation was complete. At 48 h, treated cells contained 2.5 times the level of C/EBP α mRNA found in control cells. Conversely, the levels on day 6 were nearly identical in both samples. Multiple experiments demonstrated that the onset of C/EBP α expression varied with the passage number of the cells; however, in all cases troglitazone enhanced the rate of EBP α expression. This suggests that troglitazone influences factors that regulate when C/EBP α mRNA production is induced, but not those that modulate the total amount of EBP α mRNA expressed.

Troglitazone increases basal glucose uptake in differentiated adipocytes

To determine whether troglitazone has a direct effect on glucose metabolism in the adipocytes, the 3T3-L1 tissue culture system was used to mimic adipocyte function in a controlled environment. Initial experiments were performed using 3T3-L1 cells, which showed a 35% conversion frequency without troglitazone. Cells were differentiated under standard conditions with or without troglitazone for 8 days, as described in *Materials and Methods*. On day 9, basal glucose uptake was assessed by monitoring the accumulation of [14 C]deoxyglucose within the adipocytes. As shown in Fig. 3, 0.5 μ M troglitazone treatment enhanced basal glucose uptake 9.6-fold in these cells. No effect was observed if cells were

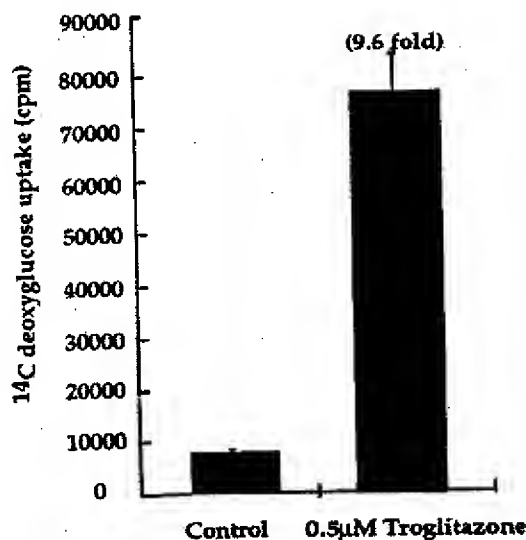


FIG. 3. Troglitazone treatment in combination with insulin increases basal glucose uptake in adipocytes. Adipocytes were differentiated using 100 nM insulin in combination with 0.25 μ M dexamethasone and 0.5 mM isomethylbutylxanthine. After 8 days, the cell [14 C]deoxyglucose uptake was monitored, as described in *Materials and Methods*. The values were obtained from duplicate samples. When assayed, the control cells exhibited 10% differentiation, whereas the troglitazone-treated cells showed 80% differentiation.

treated without the addition of the hormone cocktail (data not shown). This suggests that troglitazone increases the activity or number of functional glucose transporters per cell, or both.

Noninsulin-dependent (basal) glucose uptake is a result of transport through the membrane-associated Glut1 glucose transporter, whereas insulin-stimulated glucose uptake results from the combined activities of Glut1 and the hormone-sensitive glucose transporter, Glut4, which are associated with both the plasma membrane and microsomal compartments. Comparison studies have shown that the levels of these two glucose transporter proteins differ between preadipocytes and adipocytes (13, 20, 21) (see Fig. 7). Total cellular levels of Glut1 decline slightly with differentiation. Conversely, the amount of Glut4 transporter in the adipocyte increases from undetectable in the preadipocyte to a value 2-fold greater than that of Glut1. Comparison of the adipocyte morphology between the cultures in the glucose uptake experiment in the previous experiment indicated that the troglitazone-treated samples had a higher adipocyte/preadipocyte ratio than the control samples (data not shown). Therefore, because the number and type of glucose transporters change during adipocyte differentiation, the adipocyte/preadipocyte ratio must be equivalent between samples to accurately determine how troglitazone affects glucose transport. To do this, we repeated the previous experiment using 3T3-L1 cells that differentiated more than 95% under standard differentiation conditions without troglitazone and whose morphology and final C/EBP α mRNA levels (Fig. 2) were not significantly enhanced by troglitazone treatment. In addition, we compared the basal glucose uptake to insulin-stimulated glucose uptake, which distinguishes between Glut1 and Glut4 transporter activities. As shown in Fig. 4,

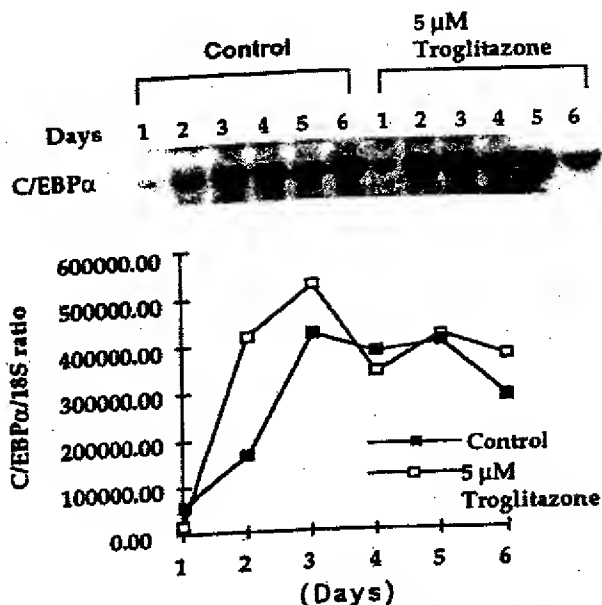


FIG. 2. Troglitazone affects the rate of C/EBP α message accumulation during differentiation, but not the overall level of message in the adipocyte. Northern analysis of total RNA collected every 24 h during adipocyte differentiation with and without 5 μ M troglitazone using a 32 P-labeled, random primed, C/EBP α probe. RNA loading was evaluated by quantitation of 18S RNA. Multiple experiments demonstrated that the onset of C/EBP α expression varied with the passage number of the cells; however, in all cases, troglitazone enhanced the rate of C/EBP α expression.

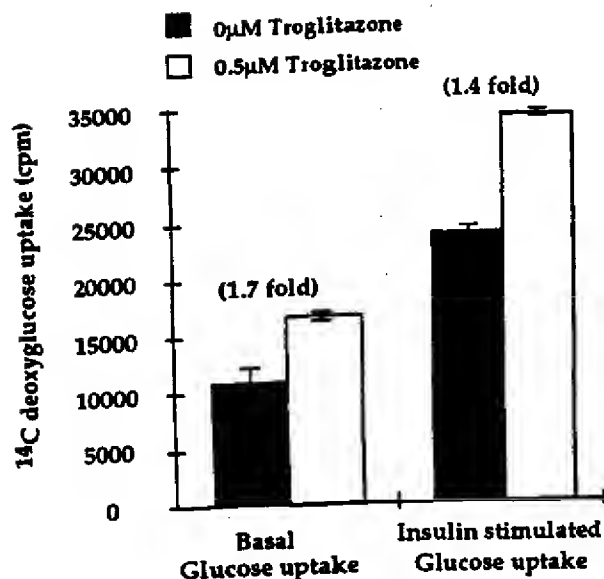


FIG. 4. Troglitazone increases basal glucose uptake in adipocytes. Adipocytes were differentiated under standard conditions in the presence or absence of 0.5 μ M troglitazone. The percentage of adipocyte differentiation in all samples was more than 95%. [¹⁴C]Deoxyglucose uptake was monitored 8 days after differentiation. For insulin-stimulated uptake, the cells were incubated for 30 min in 1×10^{-6} M insulin after a 3-h preincubation in serum-free DMEM. All values were obtained from duplicate samples.

basal glucose uptake increased 1.7-fold in response to 0.5 μ M troglitazone. Similar results (1.4-fold) were found for Glut4-dependent insulin-stimulated glucose uptake. As basal glucose uptake is a function of Glut1 transporters, and insulin-stimulated uptake results from the combination of Glut1 and Glut4 transporter activities, the fact that troglitazone enhances both basal and insulin-stimulated glucose uptake equivalently suggests that the compound only alters Glut1 transporter activity.

To further separate the effects of troglitazone on glucose uptake from those on adipocyte differentiation, adipocytes differentiated in the absence of troglitazone were treated with 0.5 or 5 μ M troglitazone for 48 h before the glucose uptake assay. As shown in Fig. 5, 0.5 and 5 μ M troglitazone treatment produced 2- and 2.7-fold increases in basal glucose transport activity. As there was no change in morphology in the cells during treatment, these data show that troglitazone can enhance basal glucose uptake activity without affecting cell differentiation.

To determine whether the enhancement of glucose transporter activity is due to an increase in transporter number or an increase in transporter function, Western analysis was performed on whole cell lysates (from cells differentiated in the presence or absence of troglitazone) using either Glut1- or Glut4-specific antibodies (Fig. 6). Again, the adipocyte/preadipocyte ratio required to eliminate the effects of differentiation on transporter levels was identical in treated and untreated samples. As previously described (13, 20, 21), Glut1 transporter levels decreased slightly with adipocyte differentiation, whereas Glut4 levels increased dramatically. Troglitazone caused a 2.3-fold increase in Glut1 protein without altering Glut4 levels. Thus, troglitazone enhances glu-

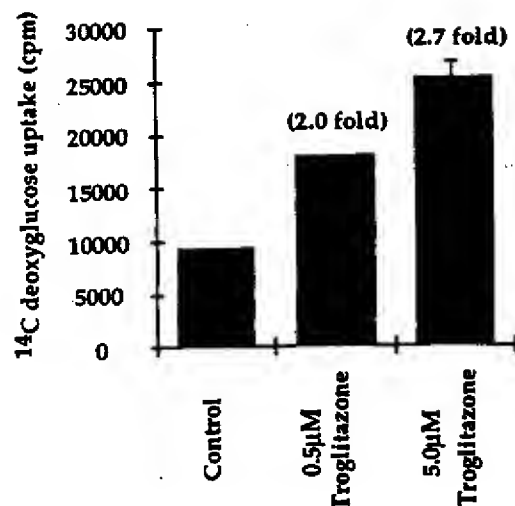


FIG. 5. Troglitazone enhances basal glucose uptake in fully differentiated adipocytes. Fully differentiated adipocytes were treated with 0, 0.5, or 5 μ M troglitazone. Basal glucose uptake was assessed 48 h after treatment. All values were obtained from duplicate samples. The percentage of adipocyte differentiation in all samples was more than 95%.

cose uptake by increasing the number of basal glucose transporters per cell. Additionally, a Glut1 RNase protection assay was performed on RNA isolated from cells differentiated in the presence or absence of 5 μ M troglitazone (Fig. 7). The Glut1/actin ratio was approximately 1.5–2 fold higher in the RNA samples isolated from troglitazone-treated cells. This directly correlates with the 2-fold increase in glucose transport and shows that troglitazone increases the number of Glut1 transporters in adipocytes.

As differentiation also affects Glut1 mRNA stability, and a previous report (12) indicated that thiazolidinediones increased mRNA stability, a Glut1 RNase protection assay was performed on RNAs isolated from adipocytes treated with 5 μ g/ml actinomycin D. Figure 8 shows that when the control cells and troglitazone cells displayed the same adipocyte/preadipocyte ratio, the rate of Glut1 mRNA decay was the same for both treated and untreated cells. Identical results were obtained from cells treated with pioglitazone, another antidiabetic thiazolidinedione (data not shown). Albeit indirectly, these data also suggest that the increase in Glut1 mRNA is due to an increase in Glut1 transcription.

Discussion

Troglitazone treatment of 3T3-L1 cells increases both the rate and percentage of adipocyte differentiation. This phenomenon is linked to the increased rate of C/EBP α accumulation in differentiating cells. Because this accumulation of C/EBP α message occurs within 24 h of drug treatment, it implies that troglitazone interacts with proteins that are present in the preadipocyte or are rapidly induced by the differentiating hormone cocktail. Recent work on adipocyte differentiation suggests that the proteins involved are members of the peroxisome proliferator-activated receptor (PPAR) family (22, 23). These nuclear receptors are activated by endogenous fatty acid or PG ligands (24–26) and in combination with C/EBP family members are believed to induce

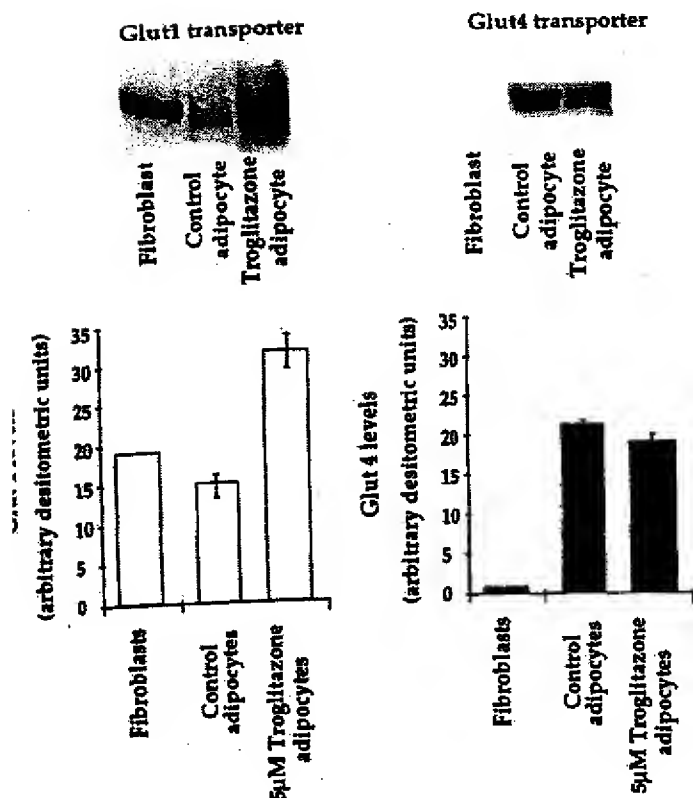


FIG. 6. Troglitazone increases the synthesis of the Glut1 transporter protein in adipocytes. Western analysis was performed using a Glut1- or Glut4-specific antibody of protein samples collected from adipocytes treated with or without 5 μ M troglitazone. The percentage of adipocyte differentiation in all samples was more than 95%. Thirty micrograms of protein were loaded per lane, as determined using Bio-Rad analysis. Histograms represent data acquired from two independent experiments.

adipocyte differentiation (27–29). Lehmann and colleagues (30) have shown that another thiazolidinedione, BRL 49653, is a ligand for PPAR γ , the adipocyte-specific PPAR family member. This suggests that by interacting with the PPARs, troglitazone initiates the cascade of transcriptional events that enhances the rate of adipogenesis.

To determine whether the increased glucose uptake in adipocytes *in vivo* is a direct effect of troglitazone or a consequence of secondary effects brought about by insulin sensitization, the effects of troglitazone were assessed in the isolated 3T3-L1 tissue culture system. Apart from the enhanced glucose uptake associated with adipocyte differentiation, troglitazone directly increased glucose uptake 2-fold in these cells. This effect was shown to be a direct result of an increased synthesis of Glut1 transporter mRNA and protein. This disagrees with previously reported results (12), which have shown that pioglitazone, another thiazolidinedione with antidiabetic activity, enhances glucose uptake in 3T3-442A adipocytes by increasing both Glut1 and Glut4 transporter mRNA and protein via mRNA stabilization. However, this study failed to distinguish between the increases in glucose uptake associated with the enhancement of adipocyte differentiation and those resulting from the direct effects of the thiazolidinedione on the adipocyte. By

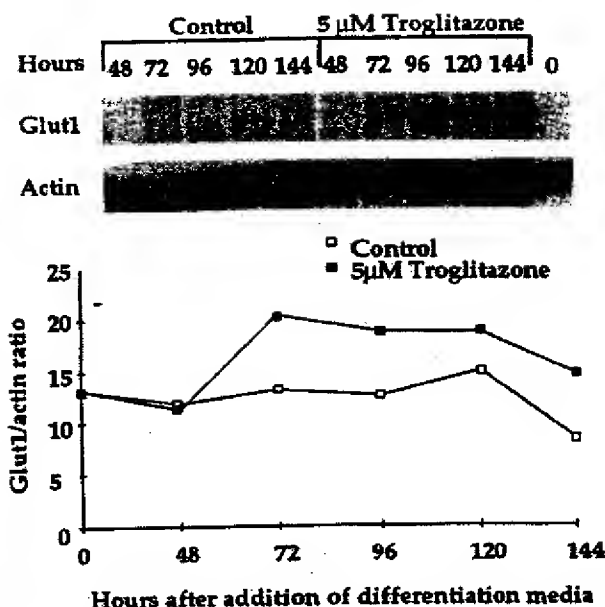


FIG. 7. The levels of Glut1 mRNA were increased in response to 5 μ M troglitazone. A RNase protection assay was performed on RNAs collected at the indicated times from cells treated with or without 5 μ M troglitazone. Ten micrograms of RNA per sample were hybridized to mouse Glut1 and β -actin probes and processed as described in Materials and Methods. Message levels were quantitated using a Molecular Dynamics PhosphorImager, and the values are represented as the Glut1/actin ratio.

controlling for the ratio of adipocytes/preadipocytes in the experiments presented here, the indirect effects of differentiation by the compound on Glut4 were eliminated, demonstrating that troglitazone enhances glucose uptake by increasing Glut1 mRNA and protein levels. Additionally, identical experiments with pioglitazone yielded similar results (data not shown).

These results provoke at least two questions. First, can a 2-fold increase in adipocyte glucose uptake account for the decreased hyperglycemia *in vivo* or must other tissues also be affected? Secondly, are the transcriptional responses involved in differentiation the same as those used in the expression of Glut1; do both require the activation of PPARs by the thiazolidinediones?

Glucose transporter number has been shown to directly affect glucose transport and blood glucose levels in animal models. Several transgenic mice have been engineered that overexpress the glucose transporters in a tissue-specific fashion (30–34). In general, overexpression of either Glut1 or Glut4 enhanced glucose transport in the targeted tissue. Enhanced transport directly correlated with decreased plasma glucose levels in both fasted and fed animals, demonstrating that enhanced transporter expression has profound effects on glucose disposal *in vivo*. Surprisingly, however, increased Glut1 expression in skeletal muscle, and hence increased muscle basal transport, resulted in resistance of Glut4 to insulin stimulation and various other stimuli, including contraction and hypoxia (32). Additionally, Glut 4 overproduction in fat cells did not protect animals from the impaired glucose tolerance induced by a high fat diet (34). Thus, although enhanced transporter synthesis can ameliorate hy-

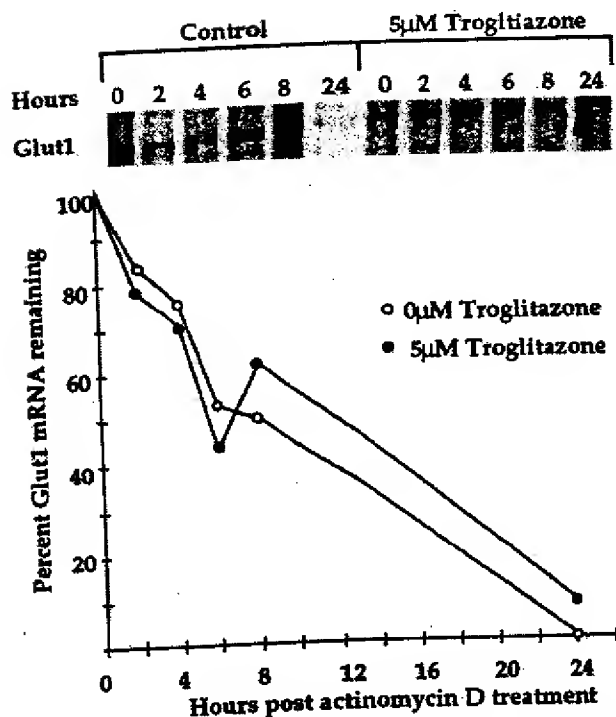


FIG. 8. Troglitazone does not alter the stability of Glut1 mRNA. RNase protection assay of Glut1 message was performed on RNA collected after actinomycin D (5 μ g/ml) treatment of adipocytes differentiated in the presence or absence of 5 μ M troglitazone. Ten micrograms of RNA were used per sample, and message levels were quantitated using a Molecular Dynamics PhosphorImager.

perglycemia, it does not appear to directly influence insulin resistance. This would imply that enhanced Glut1 synthesis in adipocytes would only represent a portion of troglitazone's antidiabetic activity. This is supported by data indicating that troglitazone influences the metabolic activities of skeletal muscle, liver, and pancreas (35–38).

The precise role of PPARs in the antidiabetic activities of the thiazolidinediones has yet to be fully explored. Clearly, the PPARs are intimately involved in lipid metabolism in a number of tissues, including adipose and liver, and are responsible for the lipid-lowering effects of the fibric acids (39). Interestingly, the lipid-lowering effects associated with thiazolidinedione treatment are similar to those of other PPAR-activating, lipid-lowering compounds (40). Moreover, as elevated lipid levels have been linked to peripheral insulin resistance, the alteration of lipid metabolism may ameliorate insulin resistance (41). However, *in vitro*, thiazolidinediones bind specifically to the PPAR γ isoform, which is mainly present in adipose tissue (22, 23). Thiazolidinediones have been shown to influence metabolism in liver, pancreas, and skeletal muscle. If all of these antidiabetic responses are to be attributed to PPAR activation, all insulin-responsive tissues must contain sufficient levels of this isoform. Alternatively, it is possible that the ligand binding specificity *in vivo* differs from that *in vitro*, and/or that the compounds stimulate the formation of ligands that activate the PPAR isoforms present in these other tissues. Future studies addressing these issues will undoubtedly reveal the role of the PPARs in the antidiabetic activity of the thiazolidinediones. However, it is clear

that the regulation of Glut1 transporter synthesis could contribute to the antidiabetic activity of troglitazone.

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The Antidiabetic Agent Pioglitazone Increases Expression of Glucose Transporters in 3T3-F442A Cells by Increasing Messenger Ribonucleic Acid Transcript Stability*

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ABSTRACT

Whereas adipocytes normally play an important role as a major site for systemic energy homeostasis, adipocyte function is markedly altered in disorders such as diabetes. In this study, we investigated the effect of pioglitazone, a novel antidiabetic agent known to lower plasma glucose in animal models of diabetes mellitus, on expression of glucose transporters GLUT1 and GLUT4 in 3T3-F442A cells. Treatment of confluent 3T3-F442A preadipocyte cultures for 7 days with pioglitazone (1 μ M) and insulin (1 μ g/ml) resulted in nearly 100% differentiation of cells to lipid-accumulating adipocytes, and such adipocytes showed a markedly increased capacity for glucose uptake. Analysis of messenger RNA transcripts encoding GLUT1 and GLUT4 glucose transporters over the 7-day differentiation period indicated time-dependent in-

creases in abundance of each type that were maximal at more than 5-fold with the combined presence of insulin and pioglitazone. In accord, GLUT1 and GLUT4 protein levels also increased to maximal levels of 10-fold and 7-fold, respectively, over those in undifferentiated preadipocytes. Increased messenger RNA half-lives from 2.2 to greater than 24 h for GLUT1 and from 1.2 to greater than 24 h for GLUT4 correlated with this induced adipocyte differentiation. Taken together, these findings indicated that pioglitazone markedly enhanced expression of cellular glucose transporters, and the mechanism for this action was mainly stabilization of transporter messenger RNA transcripts. Such increased expression of glucose transporters in adipocytes establishes the cells in a state active for glucose uptake, thus ultimately facilitating storage and metabolism as well. (*Endocrinology* 133: 352-359, 1993)

ADIPPOSE tissues play a key role in systemic energy homeostasis. Adipocytes possess hormonally regulated transport and metabolic systems allowing energy storage as triglycerides when nutrients abound or energy release during nutritional dearth. In accord with this role, a recent report indicated that adipose may be responsible for up to 30% of whole body glucose metabolism (1). Altered adipocyte function has been associated with abnormal physiological states including obesities and obesity-linked diabetes (2-4). In non-insulin-dependent diabetes mellitus, elevated blood glucose levels result from insufficient glucose uptake in adipose and muscle, a consequence of insulin resistance (5, 6).

The hyperglycemia of noninsulin-dependent diabetes mellitus can be corrected clinically by treatment of patients with oral hypoglycemic agents. Whereas presently used sulfonylurea agents appear to act principally as secretagogues to increase the availability of insulin to enhance glucose disposal (7, 8), an alternate treatment strategy could employ agents acting as insulin sensitizers, thus overcoming target tissue insulin resistance. New antidiabetic compounds belonging to the thiazolidinedione class of drugs appear to lower blood glucose in animal models of diabetes by improv-

ing insulin sensitivity in peripheral tissues (9). Treatment of insulin-resistant fatty rats or mice with the thiazolidinedione pioglitazone resulted in lowered blood glucose, triglyceride, and insulin levels (10, 11).

We and others have previously reported that thiazolidinedione agents enhanced insulin sensitivity for glucose uptake and metabolism in adipose tissues of diabetic animals (10-13). It has also been found that such agents have potent adipogenic effects on preadipocyte cell cultures (14-16). We therefore sought to more fully investigate the mechanism underlying the effect of pioglitazone to promote adipocyte differentiation with the aim of gaining insight into how such an effect could contribute to regulation of cellular glucose uptake. Findings in our present report indicate that pioglitazone treatment of 3T3-F442A preadipocytes markedly enhances expression of glucose transporters GLUT1 and GLUT4, and the mechanism for this action is a stabilization of transporter messenger RNA (mRNA) transcripts.

Materials and Methods

Cells and tissue culture

3T3-F442A fibroblasts were grown as monolayer cultures at 37°C in an atmosphere of 10% CO₂-90% air essentially as described previously (17). Subcultured cells were grown to confluence (usually 7 days) in Dulbecco's Modified Eagle's Medium (DMEM) containing glucose (4.5 g/L), bovine serum (10%), streptomycin (50 μ g/ml), penicillin (50 U/ml), Fungizone (0.25 μ g/ml), and glutamine (2 mM). Confluent cell cultures were then converted to adipocytes by culture for 7 days in

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DMEM in the presence of 10% fetal calf serum and insulin (1 $\mu\text{g}/\text{ml}$) and/or pioglitazone (1 μM). Cell differentiation was assessed by evaluating cell morphology under phase contrast microscopy; cells were considered to be adipocytes if numerous lipid droplets were observed in the cytoplasm. The cell line was used during the 10th to 20th passage after clone isolation.

Measurement of 2-deoxy-D[^{14}C]glucose uptake

For assay, 3T3-F442A cell monolayers ($\sim 10^6$ cells/17 mm well) were rinsed with PBS and incubated with 0.5 ml assay medium (DMEM, no glucose, 5 mM NaHCO_3 , 20 mM N -[2-hydroxyethyl]- N' -piperazine-[2-ethanesulfonic acid], 0.1% BSA, pH 7.4) for 15 min at 22 C. Then 0.5 μCi D-[^{14}C]glucose (stock = 57 mCi/mmol, Amersham Corp., Arlington Heights, IL) was added for an additional 15 min. After this incubation, medium was aspirated, and cells were rinsed with ice-cold PBS containing 10 mM glucose. Cells were then solubilized with 0.5 ml 0.5 N NaOH, transferred to vials, neutralized with 52 μl glacial acetic acid, and counted for radioactivity using 5 ml Ready Value scintillation fluid (Beckman, Inc., Palo Alto, CA).

Northern blot analysis of RNA for determination of GLUT1 and GLUT4 mRNA transcript abundance

Total RNA was extracted from 3T3-F442A cell monolayers essentially according to the method of Chomczynski and Sacchi (18). RNA was then size-fractionated on 1% agarose gels and transferred to nylon membranes according to the method of Fournier *et al.* (19). RNA transcripts were cross-linked to the membrane with a UV Stratilinker (Stratagene, La Jolla, CA).

Hybridization was performed using anti-sense RNA Riboprobes prepared according to the protocol of the reagent supplier (Promega Corp., Madison, WI). For GLUT1, pSPGT-1 (20) was kindly provided by Dr. Graeme Bell (Chicago, IL), and the entire 1591 base pair coding region of rat brain glucose transporter was excised using *Bgl*II and subcloned into the *Bam*HI site of pGEM-4Z. *Eco*RI was used for linearization, and T7 RNA polymerase was used for GLUT1 Riboprobe transcription. A GLUT4 construct pSM1D2 in the pBluescript KS⁺ (21) was generously provided by Dr. Morris Birnbaum (Boston, MA). For GLUT4 Riboprobe preparation, this construct was linearized with *Hind*III, and T7 RNA polymerase was used for Riboprobe preparation.

High stringency hybridizations with Riboprobes were performed by 65 C overnight incubation of membranes in glass tubes in a Hybaid Oven (National Labnet Co., Woodbridge, NJ) using procedures we have detailed previously (11). After rinses, labeled membranes were exposed at -70 C to Hyperfilm-MP (Amersham Corp., Arlington Heights, IL) with an intensifying screen. Autoradiographic bands on film were quantitated by two-dimensional densitometry using an AMBIS Optical Imaging System (San Diego, CA). RNA samples were quantitated for correction of minor loading differences by densitometry of ethidium bromide-stained 28S ribosomal bands (22) on photographic negatives (type 55 P/N film, Polaroid, Cambridge, MA).

Measurement of GLUT1 and GLUT4 mRNA stability

Confluent 3T3-F442A cells were induced to differentiate with insulin, pioglitazone, or both as described above. On day 7 of treatment, the transcription inhibiting agent Actinomycin D (Act D) was added (5 $\mu\text{g}/\text{ml}$) to treated cell cultures as well as undifferentiated control cultures, essentially as reported earlier (23–26). Total RNA was extracted from these cells at selected time points after Act D addition (0, 1, 2, 4, 6, and 24 h), and abundance of remaining GLUT1 and GLUT4 mRNA transcripts was assessed by Northern blot analyses as described above. Glucose transporter mRNA abundance data were fitted to a single exponential decay curve by nonlinear least square regression analysis. The estimated first-order rate constant was used to calculate the mRNA half-life. Since it was suggested that Act D may have nonspecific effects with long-term (24 h) treatments (24), glucose transporter mRNA half-life calculations were based on changes in mRNA abundance over only the first 6 h of Act D treatment.

Western blot analysis of glucose transporter proteins

Total particulate membrane proteins were prepared as previously described (27). Briefly, cells were washed with PBS and scraped into homogenization buffer [20 mM Tris-HCl, 255 mM sucrose, 1 mM EDTA, 1 mM phenylmethyl sulfonylfluoride, 10 U/ml Trasylol] and were homogenized with 10 pulses by a Tekmar TissueMizer (Tekmar Inc., Cincinnati, OH). A total membrane fraction was prepared by centrifugation of the homogenate at 200,000 $\times g$ at 4 C. Protein concentrations were determined by the Bradford assay (28) using BSA as a standard. Initial Western blot analyses revealed diffuse bands of GLUT1 and GLUT4 proteins, possibly due to heterogeneous glycosylation of the transport proteins. Therefore, samples were routinely treated with peptide *N*-glycosidase F (1 U/100 μg protein) in a buffer containing 20 mM sodium phosphate, pH 7.5, 10 mM EDTA, 1.7% Triton X-100, and 1 mM phenylmethyl sulfonylfluoride for 48–72 h at 37 C to remove sugar residues (23). Treated protein samples were then mixed with one fourth volume of 4 \times electrophoretic sample buffer [200 mM Tris-HCl at pH 6.8, 400 mM dithiothreitol, 8% sodium dodecyl sulfate, 40% glycerol, 0.4% bromophenol blue] and stored at -20 C. Samples were thawed and loaded in parallel onto two discontinuous 12% polyacrylamide gels, and size-fractionated according to the method of Laemmli (29) using a Mini-Protein II Dual Slab Cell (Bio-Rad, Richmond, CA). The amount of protein loaded (10 $\mu\text{g}/\text{lane}$ for GLUT1 and 60 $\mu\text{g}/\text{lane}$ for GLUT4) was determined empirically to be within the linear response range for the system used. Proteins separated on each gel were electrophoretically transferred to Immobilon PVDF membranes (Millipore Corp., Bedford, MA) using a Mini-Transblot electrophoretic transfer cell (Bio-Rad). One membrane was stained for total protein (0.1% Coomassie R-250, 40% methanol, 10% acetic acid). The second membrane was immunostained for GLUT1 or GLUT4 using a double antibody system and the Immuno-Blot Assay Kit (Bio-Rad). The supplier's instructions were followed except 5% BSA was used for membrane blocking. The primary antibodies (RaGLUTRANS for GLUT1 and RaRGT for GLUT4, East Acres Biologicals, Southbridge, MA) were diluted 1:3000 and 1:2000, respectively, and the alkaline phosphatase-conjugated second antibody (GAR-AP, Bio-Rad) was diluted 1:3000 for use. Resulting signals were quantitated with the reflective mode of a Model 620 Video Densitometer (Bio-Rad). Sample loading corrections were made based on densitometry data from the Coomassie-stained membrane.

Data analysis

Statistical analysis was performed using SAS version 6 (SAS Institute, Inc., Cary, NC). All hypothesis tests were two-sided and were considered significant if the *P* value was less than or equal to 0.05.

Results

Induction of preadipocyte differentiation and glucose transport by insulin and pioglitazone

Treatment of confluent 3T3-F442A cells for 7 days with insulin (1 $\mu\text{g}/\text{ml}$), pioglitazone (1 μM), or both agents in the presence of 10% fetal calf serum resulted in conversion of cells into lipid-accumulating adipocytes. Whereas some cells differentiated into adipocytes by either treatment alone (insulin, 60%; pioglitazone, 80%), nearly complete differentiation (95%) was achieved with both agents together. Such cellular differentiation was associated with markedly increased capacity for glucose transport. Treatment of 3T3-F442A cells for 7 days with insulin or pioglitazone resulted in basal glucose transport levels that were 22- and 30-fold increased compared to untreated fibroblasts (Table 1). Together, both agents appeared to act additively for a maximal enhancement of 61-fold at day 7. Age-matched, undifferentiated cells maintained in growth medium showed no

TABLE 1. P and I-induced increases in glucose transport activity in differentiating 3T3-F442a Cells

Cell treatment	Glucose uptake (dpm/cell \pm SEM)
Control, day 0	0.0030 \pm 0.00007
Control, day 7	0.0013 \pm 0.00031
Insulin	0.0670 \pm 0.0076
Pioglitazone	0.0887 \pm 0.015
Insulin + pioglitazone	0.1830 \pm 0.037

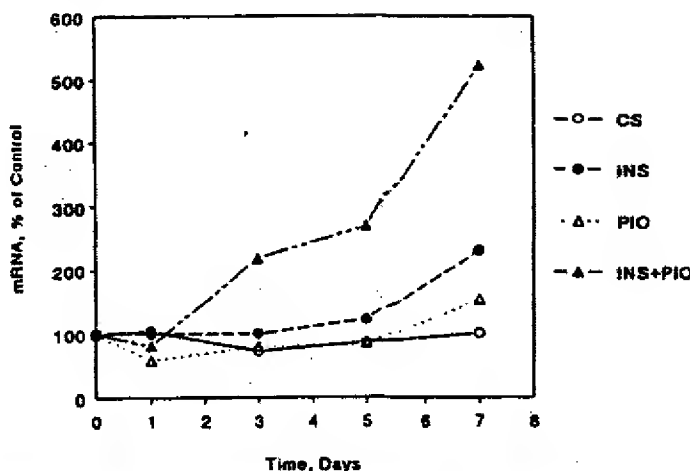
Confluent monolayer cultures of preadipocytes in DMEM (day 0) were induced to differentiate by treatment with insulin and/or pioglitazone. Preadipocyte cells maintained for 7 days in DMEM without added insulin and/or pioglitazone also served as controls. After treatment for 7 days, glucose transport activity was measured by uptake of 2-deoxy-D¹⁴C glucose as described in *Materials and Methods*. Since the radiolabeled glucose has a specific activity of 300 mCi/mmol, there are 1.5 f/dpm. Thus, maximal basal transport was approximately 0.3 fmol/cell for cells differentiated in medium containing insulin and pioglitazone.

increase in glucose transport over the 7-day interval, and actually declined about 60%, likely an indication of quiescence.

Insulin- and pioglitazone-enhanced expression of glucose transporters in 3T3-F442A cells

To assess whether such increased glucose transport activity could be explained by amplified expression of glucose transporters in adipocytes, we analyzed the levels of GLUT1 and GLUT4 mRNA abundance. When assessed by Northern blotting, the abundance of mRNA transcripts encoding GLUT1 and GLUT4 glucose transporters increased in a time-dependent manner (Figs. 1, 2). Whereas 7-day treatment with either insulin (1 μ g/ml) or pioglitazone (1 μ M) increased GLUT1 mRNA abundance by 2.3- and 1.5-fold, respectively, above the level in undifferentiated cells (day 0), insulin and pioglitazone together acted synergistically to increase this message by almost 6-fold (Fig. 1). GLUT1 mRNA levels did not change in age-matched undifferentiated cells maintained in growth medium over the same interval. In contrast to the observed synergistic treatment effects on GLUT1 mRNA levels, GLUT4 mRNA abundance was increased to similar levels above undifferentiated cells (day 0) reaching 3.8-, 4.6-, and 5.2-fold elevation by respective treatments for 7 days with insulin, pioglitazone, or both (Fig. 2). Whereas these values for GLUT4 mRNA abundance reflected an overall increase at day 7, a small decline was observed at this time point in some experiments with combined treatment by insulin and pioglitazone (Fig. 2, lower). Such results indicate that some down-regulation may occur in the final differentiated state.

These changes in GLUT1 and GLUT4 glucose transporter mRNA levels were accompanied by changes in levels of the encoded transporter proteins as determined by Western blotting. Whereas either insulin or pioglitazone treatment appeared to increase GLUT1 protein levels on day 7 of differentiation by 2.3- and 3.5-fold respectively, the agents together seemed to act synergistically to increase protein levels by almost 10-fold above those of age-matched undifferentiated cells (Fig. 3). In contrast to the observed synergistic



GLUT1 mRNA

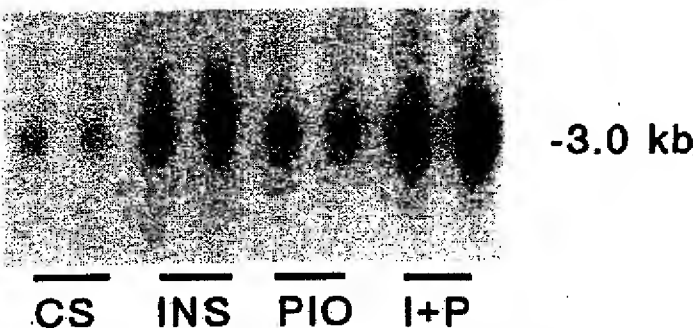
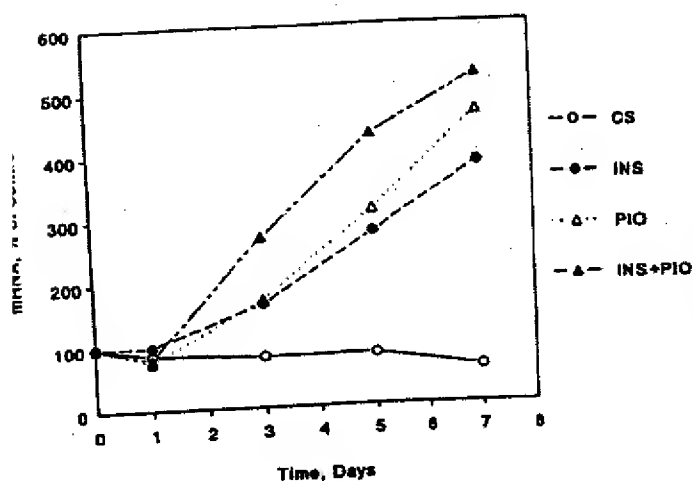


FIG. 1. Effect of insulin and pioglitazone treatment on GLUT1 mRNA abundance. *Upper*, Time-dependent increases in abundance of GLUT1 mRNA during differentiation of 3T3-F442A cells induced by insulin (INS), pioglitazone (PIO), or both. Cells were seeded, grown to confluence, and differentiated as described in *Materials and Methods*. Untreated age-matched fibroblasts maintained in DMEM containing only 10% calf serum were used as control (CS). Total RNA was isolated at indicated time points, and samples (10 μ g/lane) were electrophoretically size-fractionated on agarose gels. Northern blots were hybridized to Riboprobes specific for rat GLUT1 mRNA. Autoradiographic bands were quantitated by densitometry and normalized for minor loading differences as described in *Materials and Methods*. Data represent mean values for $n = 5-6$ determinations. A three factor analysis of variance (ANOVA; insulin, pioglitazone, time) with interactions showed a significant effect on GLUT1 mRNA abundance from combined treatment with I and P ($P < 0.001$). *Lower*, Representative Northern blot showing GLUT1 mRNA abundance in control (CS) and treated (INS, PIO, I + P) cells on day 7 of differentiation.

effect on GLUT1 protein, GLUT4 protein levels increased by about 2.2-, 6.6-, and 4-fold by insulin, pioglitazone, or both, respectively (Fig. 4).

Increased stability of glucose transporter messages

We next investigated possible effects of insulin and pioglitazone on the stability of GLUT1 and GLUT4 mRNAs. Fibroblast 3T3-F442A cells were induced to differentiate by treatment with insulin (1 μ g/ml), pioglitazone (1 μ M), or both for 7 days. The transcription inhibiting agent, Act D (5 μ g/ml), was added to differentiated cells on day 7 or to undifferentiated control cells just before such cells reached conflu-



GLUT4 mRNA

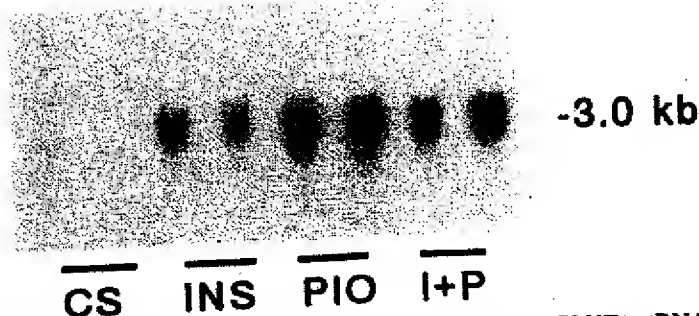
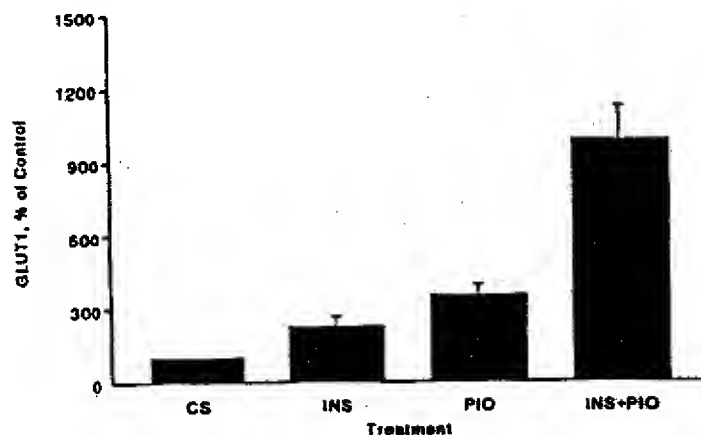


FIG. 2. Effect of insulin and pioglitazone treatment on GLUT4 mRNA abundance. *Upper*, Time-dependent increases in GLUT4 mRNA abundance in 3T3-F442A cells during differentiation. Cells were grown to confluence, and differentiated in the presence of insulin (INS), pioglitazone (PIO), or both (INS + PIO) as described in *Materials and Methods*. Untreated, age-matched fibroblasts maintained in DMEM containing only 10% calf serum were used as control (CS). Total RNA was isolated. Northern blots were prepared as described for Fig. 1, and membranes were hybridized with Riboprobe transcripts specific for rat GLUT4. Quantitation of resultant autoradiographs was done as described in *Materials and Methods*. Data represent mean values for $n = 5-6$ determinations. A three factor ANOVA (insulin, pioglitazone, time) with interactions showed significant effects on GLUT4 mRNA abundance by INS ($P = 0.001$) or PIO ($P < 0.0001$). *Lower*, Northern blot showing GLUT4 mRNA abundance in control (CS) and treated (INS, PIO, I + P) cells on day 7 of differentiation.

ence, and total RNA was extracted from cells at indicated times after addition of the transcription inhibitor (0, 1, 2, 4, 6, and 24 h). Even after 24-h treatment with Act D, cell membranes remained intact and appeared refractile, and there was no visible sloughing of cells from the plates. The abundance of remaining mRNA transcripts for each of the glucose transporter genes was assessed on Northern blots. Differentiation of 3T3-F442A cells increased the GLUT1 mRNA half-life from about 2.2 h in control undifferentiated cells to about 5.7, 3.6, and greater than 24 h in adipocytes differentiated by treatment with insulin, pioglitazone, or both, respectively (Fig. 5, Table 2). The stabilization of GLUT1 mRNA appeared to account for corresponding increases in GLUT1 mRNA abundance. Similarly, GLUT4



GLUT1 Protein

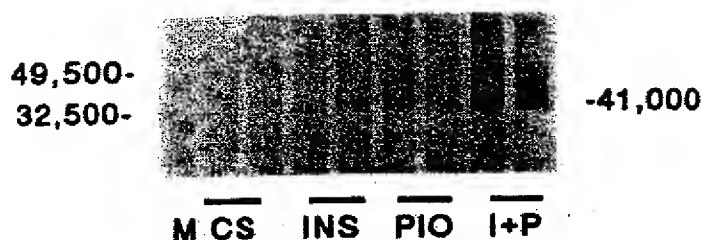


FIG. 3. Effect of insulin and pioglitazone treatments on GLUT1 protein abundance. *Upper*, Increased GLUT1 protein abundance on differentiation of 3T3-F442A cells. Confluent 3T3-F442A cells were differentiated with I (INS), P (PIO), or both (INS + PIO) as described in *Materials and Methods*. On day 7, cellular membranes were isolated from undifferentiated (CS) or differentiated (INS-, PIO-, or INS + PIO-treated) cells, and protein (10 μ g/lane) was electrophoresed and immunoblotted for GLUT1 detection using the rabbit anti-GLUT1 primary antibody (see *Materials and Methods*). Resulting colorimetrically stained bands were quantitated by densitometry and normalized for minor loading differences. Data are expressed as percent of control and represent mean values \pm SEM for $n = 8$ determinations. With background subtraction, the mean basal value was 0.20 ± 0.06 arbitrary density units. Two factor ANOVA showed significant effects on GLUT1 protein by INS ($P = 0.014$), PIO ($P = 0.015$), and INS + PIO ($P < 0.0001$). *Lower*, Representative immunoblot showing expression of GLUT1 protein in selected samples from control (CS) and treated (INS, PIO, I + P) cells on day 7 of differentiation. Molecular weight markers are indicated in lane M; the GLUT1 band ran as M, = 41,000.

mRNA half-life increased from about 1.2 h in undifferentiated control cells to about 14.2, 10.3, and greater than 24 h in cells differentiated with insulin, pioglitazone, or both, respectively (Fig. 5, Table 2). Again, GLUT4 mRNA stabilization appeared to account for observed increases in mRNA abundance.

As shown in Fig. 6, we were able to quantitate differential expression of transporter messages in cells undergoing different treatments by differentially exposing the Northern blots to film. This allowed calculation of message half-lives even when transcripts were present only at relatively low levels.

Discussion

Pioglitazone, 5-[4-(2-(5-ethyl-pyridyl)ethoxy)-2,4-thiazolidinedione], is an antidiabetic agent that has been shown to

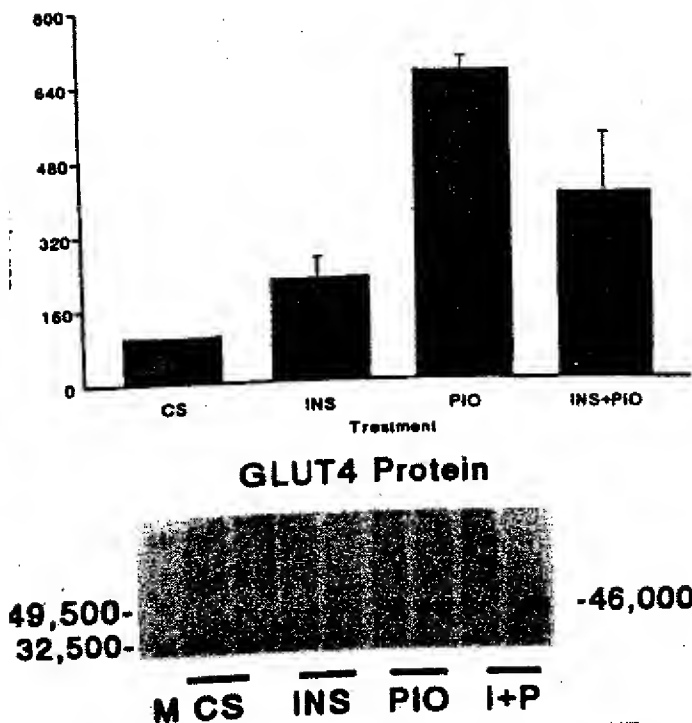


FIG. 4. Effect of insulin and pioglitazone treatments on GLUT4 protein abundance. *Upper*, Increased GLUT4 protein levels on differentiation of 3T3-F442A cells. Cell culture, treatment conditions, protein extraction, and Western blot analyses were performed as described in Fig. 3 legend, except that 60 μ g/lane total protein were loaded for detection using the rabbit anti-GLUT4 primary antibody. Data are expressed as percent of control and represent mean \pm SEM for $n = 10$ determinations. After background subtraction, the mean basal value was 0.57 ± 0.11 arbitrary density units. Two factor ANOVA showed significant effects on GLUT4 by INS ($P = 0.015$), PIO ($P = 0.007$), and INS + PIO ($P = 0.0003$). *Lower*, Representative immunoblot showing increased levels of GLUT4 protein in 7 day-treated (INS, PIO, I + P) compared to untreated control (CS) cells. GLUT4 protein appears as a stained band with apparent M, of 46,000. In control cells (CS), a nonspecific band of apparent M, 60,000 cross-reacted with the secondary antibody.

ameliorate hyperglycemia in animal models of noninsulin-dependent diabetes mellitus (10, 11, 30). The purpose of our study was to further probe cellular action mechanisms underlying the antidiabetic effects of pioglitazone. Our prior findings indicated that the antidiabetic agent pioglitazone acted as a potent accelerator of adipocyte differentiation of 3T3-F442A cells (14). This was evidenced by our demonstration that treatment of fibroblast-like preadipocytes with pioglitazone led to expression of fat-specific genes along with acquisition of the morphological appearance of lipid-accumulating adipocytes with concomitant increases in triglyceride accumulation (14). In the present study, we showed that such pioglitazone-treated cells showed an increased capacity for glucose uptake, with associated increases in GLUT1 and GLUT4 proteins. We further measured increased levels of mRNA transcripts encoding these glucose transporters, and found that these increases corresponded with markedly enhanced stability of both GLUT1 and GLUT4 mRNA messages. Such increased expression of glucose transporters in adipocytes established the cells in a state active for glucose

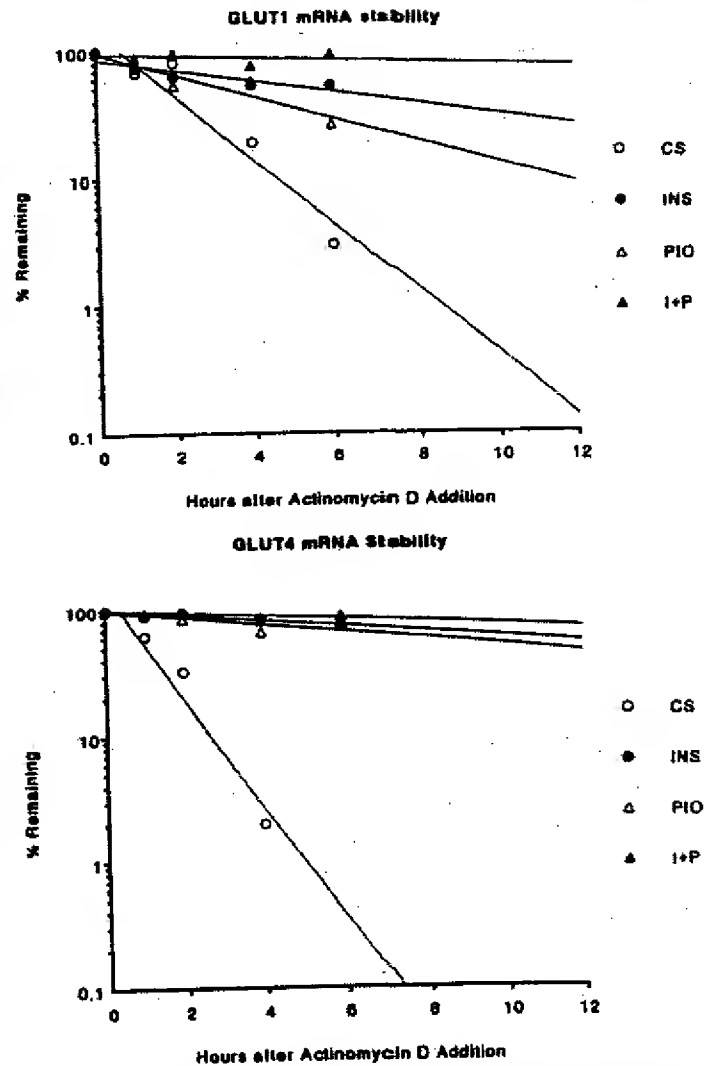


FIG. 5. *Upper*, Enhanced GLUT1 mRNA stability associated with treatment of 3T3-F442A cells with insulin (INS), pioglitazone (PIO), or both (I + P). *Lower*, Enhanced GLUT4 mRNA stability associated with treatment of 3T3-F442A cells with I, P, or both. Confluent 3T3-F442A cells were differentiated with INS, PIO, or I + P for 7 days as described in *Materials and Methods*. On day 7 of treatment, Act D (5 μ g/ml) was added to differentiated (INS, PIO, I + P) and undifferentiated (CS) control cells, and total RNA was extracted from cells at indicated time points (0, 1, 2, 4, 6, and 24 h). Abundance of mRNA was assessed by Northern blotting analysis as described earlier. Data are expressed as percent of mRNA remaining after Act D treatment relative to the levels before the treatment time 0. Each data point represents a mean value for $n = 2$ determinations. Calculated mRNA half-lives are shown in Table 2.

uptake, thus ultimately facilitating glucose storage and metabolism as well.

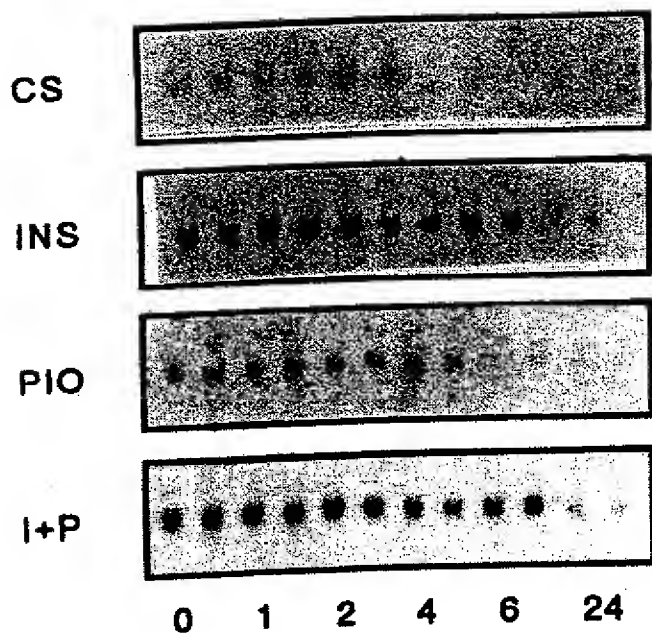
Results of our present study showed that the differentiation of 3T3-F442A cells by treatment with insulin and pioglitazone was accompanied by strikingly increased capacity for basal glucose transport (60-fold) compared to that for fibroblast-like preadipocytes. Facilitated diffusion of glucose across the plasma membrane of adipocytes is known to be mediated by two glucose transporter proteins, i.e. GLUT1

ABLE 2. Calculated mRNA half-lives in undifferentiated and differentiated 3T3-F442A cells

Treatment	mRNA half-life (h)			
	CS	INS	PIO	INS + PIO
Transporter				
GLUT1	2.2	5.7	3.6	>24
GLUT4	1.2	14.3	10.3	>24

Data from the experiment involving Act D treatment (Fig. 5) were fitted to a single exponential decay curve by nonlinear least square regression analysis. The estimated first-order rate constant was used to calculate the mRNA half-life.

GLUT1 mRNA Stability



Hours after Actinomycin D Addition

FIG. 6. Autoradiographs of Northern blots probed for GLUT1 mRNA transcript abundance after D treatment. Experimental samples were prepared as described in Fig. 5. Data shown represent duplicate experimental points, except for the time 0 INS, where only a single sample is shown. Hours of film exposure to the radiolabeled blot were 40, 18, 24, and 8 for CS, INS, PIO, and INS + PIO, respectively. Some films required longer exposure intervals for detection of low level transcripts.

and GLUT4 (31). Multiple mechanisms exist by which hormones and other factors control the rate of cellular glucose uptake via these transporters. These include the rapid translocation of preexisting transporters from an intracellular pool to the plasma membrane, modulation of the intrinsic activity of preexisting plasma membrane glucose transporters, and stimulation of the synthesis of new transporters (32). We clearly observed increased transporter synthesis, since our results demonstrated increases in the expression of both GLUT1 and GLUT4 transporters on mRNA and protein

levels (5- to 10-fold maximal enhancement for each). These results are in agreement with previous studies in which GLUT1 and GLUT4 mRNA and protein levels were shown to increase during differentiation of 3T3 preadipose cells (27, 33). Our observed increase in glucose transporter activity appeared to be somewhat greater in proportion than the combined increases in the synthesis of the two transporter proteins. This observation indicated that another mechanism, such as increased intrinsic activity of transporters, may also contribute to the effect. Such regulation of intrinsic transporter activity has previously been reported (34, 35). Inhibition of 3T3-L1 adipocyte protein synthesis by anisomycin, for instance, appeared to stimulate glucose transport primarily by enhancing the intrinsic catalytic activity of cell surface GLUT1, and to a lesser extent GLUT4 proteins (34). Further, treatment of 3T3-L1 preadipocytes with tumor necrosis factor- α reportedly increased glucose transport and GLUT1 transporter intrinsic activity (35). Alternatively, it is possible that the apparent difference in glucose transport activity and glucose transporter protein levels may be a consequence of our presentation of data as fold-enhancement relative to low level controls. As such, we may have some differences in detection sensitivity rather than absolute differences in the magnitude of changes.

Increased mRNA abundance can be attributed to enhanced RNA transcription and/or increased message stability. It was previously established that increases in the steady state level of several mRNAs during differentiation were accompanied by activation of specific gene transcription (36-39). This was particularly shown for mRNAs whose abundance was increased markedly (20- to 100-fold) during differentiation, including the *ap2* and glycerolphosphate dehydrogenase genes (36). The same report showed no significant changes in the rates of transcription of mRNAs for which abundance was more moderately altered (2- to 4-fold) during differentiation, such as those encoding fructose-1,6-biphosphate, β -actin, and β -tubulin (36). In any case, most adipocyte mRNAs were far more abundant than would be predicted by their increased nuclear transcription rates. When increases in steady state mRNA levels cannot be attributed to changes in transcription, other levels of control such as mRNA stability likely contribute to the relative abundance of mRNAs during adipocyte differentiation. Since we observed moderate increases in GLUT1 and GLUT4 mRNA abundance (about 5-fold) with adipocyte differentiation, we compared mRNA half-lives in undifferentiated and differentiated cells using Act D (a transcription inhibitor) chase experiments. Differentiation of 3T3-F442A cells by insulin and/or pioglitazone dramatically increased the mRNA half-lives for GLUT1 and GLUT4 above their values in undifferentiated cells, i.e. from 1-2 h up to greater than 24 h. Such stabilization of these mRNAs with adipocyte differentiation correlated well with increases in the mRNA steady state levels. It is interesting to note that Actinomycin D itself has been reported in a few instances to have mRNA stabilizing effects (40-42). It would therefore be possible to extend our observations by conducting experiments using different transcription inhibitors such as 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole or thiolutin, or using a different method such as [3 H]uridine pulse to

rify message stability. However, our observed dramatic increase in the stability of transcripts encoding glucose transporters in adipocytes versus fibroblasts seems likely to remain, though there may be some differences in the absolute calculated half-lives.

Our findings represent the first study that directly associates increased steady state mRNA levels during adipocyte differentiation with increased mRNA stability. This process was, however, predicted earlier based on observed increases in mRNA transcript levels that could not be explained by increased gene transcription (36–38). Regulation of mRNA stability in such cells has, in fact, been reported for other conditions. Treatment of 3T3-F442A mature adipocytes with retinoic acid, for instance, specifically decreased the adipisin mRNA level (43). For such studies, the rate of adipisin gene transcription remained unchanged, whereas the half-life of adipisin mRNA was greatly shortened in retinoic acid-treated adipocytes as compared with untreated cells (37.6–7.3 h). Another example is the induction of GLUT1, as well as other immediate-early growth-related protooncogenes, in 3T3-L1 fibroblasts by treatment with tumor necrosis factor- α (26, 35) and 8-bromo-cAMP (44). Whereas transcriptional activation of immediate-early genes correlated well with subsequent accumulation of their respective mRNAs, increased GLUT1 mRNA was due to an apparent increase in the stability of this message (45 min to several hours) without changes in its transcription. Another study showed that increased GLUT1 mRNA abundance by chronic exposure of L6 myocytes to insulin was due to increased transcription as well as prolonged half-life (2–5 h) (45).

In general, the stabilization/destabilization of mRNAs in response to biological and pharmacological stimuli has been recognized as an important posttranscriptional step for regulation of gene expression (46–49). Despite that, the mechanisms underlying such processes, including the signals that trigger mRNA degradation or stabilization, the structural elements of the RNA that are recognized by degradative enzymes or stabilization factors, as well as the enzymes or other *trans*-acting factors themselves, are largely unknown (46, 49–52). Interestingly, a recent report indicated that the 3'-untranslated region of GLUT1 mRNA contains a single copy of the destabilizing AUUUA motif in the context of an AU-rich region (26). The stability of GLUT1 mRNA was found to be partially controlled by its interaction with a sequence-specific mRNA binding protein, the adenosine-uridine binding factor which was speculated to mediate mRNA stabilization by blocking the AU-destabilizing motifs (26). We therefore propose that increasing mRNA abundance during differentiation by increasing message stability presents an interesting phenomenon awaiting further examination. Future efforts should be particularly directed toward identifying common mRNA sequences that may function as stabilizing elements in the differentiation-induced mRNAs as well as identifying their regulatory binding proteins.

Acknowledgments

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L-Glutamic Acid γ -Monohydroxamate

A POTENTIATOR OF VANADIUM-EVOKED GLUCOSE METABOLISM *IN VITRO* AND *IN VIVO**

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We report that the vanadium ligand L-Glu(γ)HXM potentiates the capacity of free vanadium ions to activate glucose uptake and glucose metabolism in rat adipocytes *in vitro* (by 4–5-fold) and to lower blood glucose levels in hyperglycemic rats *in vivo* (by 5–7-fold). A molar ratio of two L-Glu(γ)HXM molecules to one vanadium ion was most effective. Unlike other vanadium ligands that potentiate the insulinomimetic actions of vanadium, L-Glu(γ)HXM partially activated lipogenesis in rat adipocytes in the absence of exogenous vanadium. This effect was not manifested by D-Glu(γ)HXM. At 10–20 μ M L-Glu(γ)HXM, lipogenesis was activated 9–21%. This effect was approximately 9-fold higher ($140 \pm 15\%$ of maximal insulin response) in adipocytes derived from rats that had been treated with vanadium for several days. Titration of vanadium(IV) with L-Glu(γ)HXM led to a rapid decrease in the absorbance of vanadium(IV) at 765 nm, and ^{51}V NMR spectroscopy revealed that the chemical shift of vanadium(IV) at -490 ppm disappeared with the appearance of a signal characteristic to vanadium(V) (-530 ppm) upon adding one equivalent of L-Glu(γ)HXM. In summary, L-Glu(γ)HXM is highly active in potentiating vanadium-activated glucose metabolism *in vitro* and *in vivo* and facilitating glucose metabolism in rat adipocytes in the absence of exogenous vanadium probably through conversion of trace intracellular vanadium into an active insulinomimetic compound. We propose that the active species is either a 1:1 or 2:1 L-Glu(γ)HXM:vanadium complex in which the endogenous vanadium(IV) has been altered to vanadium(V). Finally we demonstrate that L-Glu(γ)HXM- and L-Glu(γ)HXM-vanadium-evoked lipogenesis is arrested by wortmannin and that activation of glucose uptake in rat adipocytes is because of enhanced translocation of GLUT4 from low density microsomes to the plasma membrane.

Intensive studies have been carried out during the last two decades on the insulinomimetic effects of vanadium (1–4). Va-

vanadium salts mimic most of the effects of insulin on the main target tissues of the hormone *in vitro* and also induce normoglycemia and improve glucose homeostasis in insulin-deficient (5–7) and insulin-resistant diabetic rodents *in vivo* (5–8). On the basic research frontier, data continue to accumulate showing that vanadium salts manifest their insulin-like metabolic effects through alternative pathways not involving insulin receptor tyrosine kinase activation or phosphorylation of insulin receptor substrate 1 (9–19). The key events of this backup system appear to involve inhibition of protein-phosphotyrosine phosphatases and activation of nonreceptor protein-tyrosine kinases (20–23).

Vanadium salts are seriously considered as a possible treatment for diabetes, and several clinical studies have already been performed. In those studies, because of its toxicity, only low doses of vanadium (2 mg/kg/day) were used. Although ~ 20 -fold lower than doses used in most animal studies, several beneficial effects were observed and documented (24–26). Any manipulation to elevate the insulinomimetic efficacy of vanadium without increasing its toxicity is of major clinical interest for the future care of diabetes (reviewed in Ref. 27).

Organically chelated vanadium compounds, such as vanadium-acetylacetonate and vanadium-RL-252¹ are more potent than free vanadium in facilitating insulin-like effects in rat adipocytes (28, 29). Similarly, chelated vanadium compounds such as bis(maltolato)oxovanadium and bis(picolinato)oxovanadium are more effective than free vanadium in reducing circulating glucose levels in hyperglycemic streptozocin-treated rats (30–33).

In the wake of these findings, we have continued our search for more effective vanadium binding agents. Of special interest to us were vanadium chelators that synergize with vanadium both *in vivo* (i.e. in streptozocin rats) and *in vitro* (i.e. in isolated rat adipocytes) and therefore enable us to gain insight into the basic mechanism(s) by which such compounds potentiate the insulinomimetic activity of vanadium. Specifically, we have studied hydroxamic acid derivatives. These compounds are involved in the microbial transport of iron and are therefore applied therapeutically in conditions of iron deficiency (34). They are also inhibitors of urease activity and have been used in the treatment of hepatic coma. Monoamino acid hydroxamates are simple, nontoxic derivatives of amino acids. D-Aspartic acid β -hydroxamate was shown to have antitumoral activity on murine leukemia L5178Y, both *in vitro* and *in vivo*, and is

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¹ The abbreviations used are: RL-252, $[(\text{CH}_3)_2\text{C}(\text{CH}_2\text{O}(\text{CH}_2)_4\text{CO-NHCH}(\text{tBu})\text{CONOHCH}_2)_2]$; L-Glu(γ)HXM, L-glutamic acid γ -monohydroxamate; GLUT4, glucose transporter 4; PM, plasma membranes; LDM, low density microsomes; BSA, bovine serum albumin; VOCl_2 , vanadyl dichloride; NaVO_3 , sodium metavanadate.

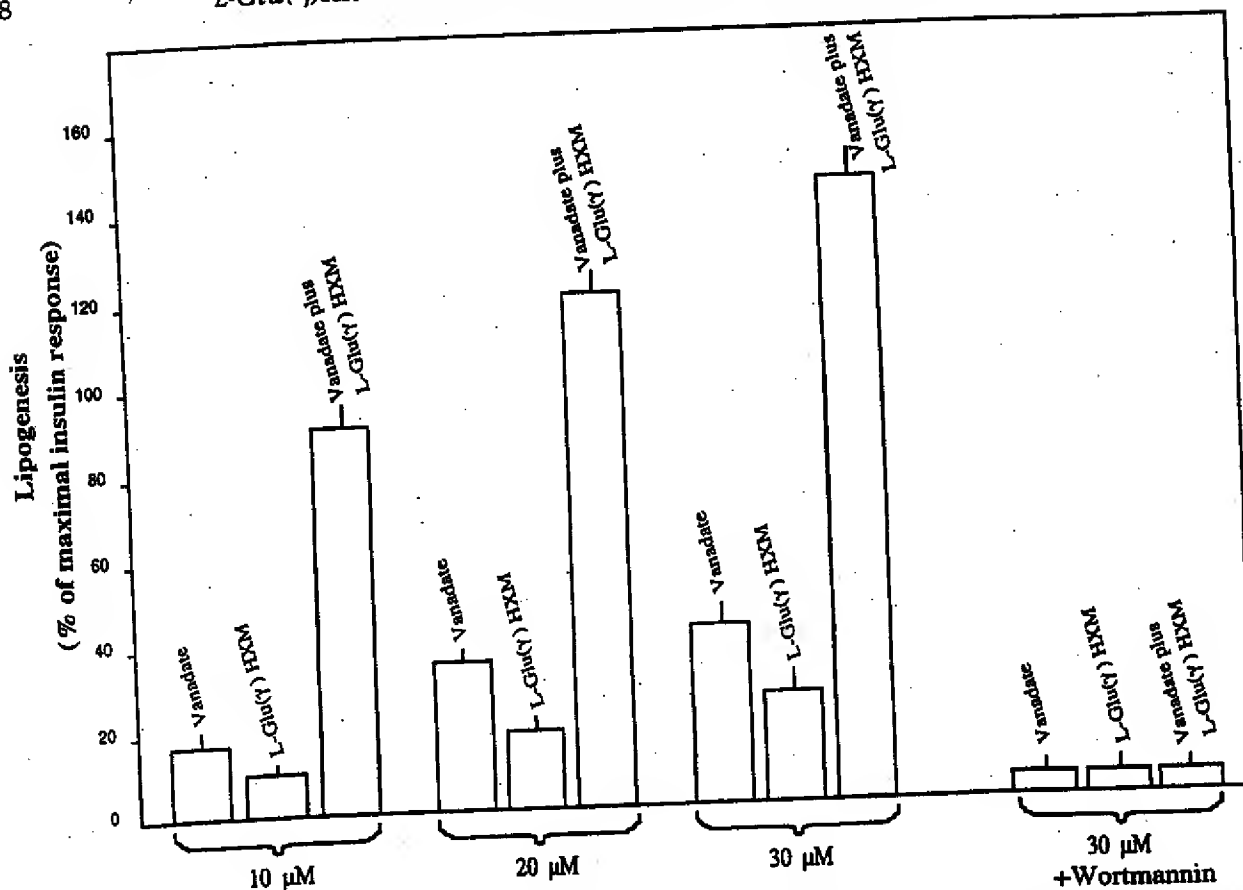


FIG. 1. Increase in the lipogenic capacity of vanadium(V) following the addition of L-glutamic acid(γ)monohydroxamate. Freshly prepared rat adipocytes (3×10^6 cells/ml) suspended in KRB buffer, pH 7.4, containing 0.7% BSA were preincubated for 10 min with the indicated concentrations of NaVO_3 , free Glu(γ)HXM, and a 1:1 complex of L-Glu(γ)HXM- NaVO_3 . The cells were then supplemented with [U - ^{14}C]glucose, and lipogenesis was performed for 2 h at 37°C . Radioactivity incorporated into extracted lipids was then determined. Maximal response (100%) is that obtained in the presence of 17 nM insulin.

active against Friend leukemia cells *in vitro* as well (35). L-Glu(γ)HXM is cytotoxic against L1210 cells in culture and remarkably antitumoral against L1210 leukemia and B16 melanoma *in vivo* (35, 36).

EXPERIMENTAL PROCEDURES

Materials—D-[U - ^{14}C]glucose and 2-deoxy-D-[G - ^3H]glucose were purchased from NEN Life Science Products. Collagenase type I (134 units/mg) was obtained from Worthington. Porcine insulin was purchased from Eli Lilly Co. (Indianapolis, IN). Phloretin, 2-deoxyglucose, L-glutamic acid(γ)monohydroxamate, L-aspartic acid(β)monohydroxamate, glycine hydroxamate, L-isoleucine(α)hydroxamate, and L-tyrosine(α)hydroxamate were purchased from Sigma. RL-252 was prepared and characterized as described earlier (28).

Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) contained 110 mM NaCl, 25 mM NaHCO_3 , 5 mM KCl, 1.2 mM KH_2PO_4 , 1.3 mM CaCl_2 , 1.3 mM MgSO_4 . Krebs-Ringer bicarbonate HEPES (KRBH) buffer (pH 7.4) consisted of 117 mM NaCl, 10 mM NaHCO_3 , 1 mM CaCl_2 , 1 mM MgSO_4 , 4 mM KH_2PO_4 , 30 mM HEPES. All other chemicals and reagents used in this study were of analytical grade.

Streptozocin-treated Rats—Diabetes was induced by a single intravenous injection of a freshly prepared solution of streptozocin (55 mg/kg body weight) in 0.1 M citrate buffer, pH 4.5 (9). The effect of the L-Glu(γ)HXM-vanadium complex on blood glucose level was determined 8 days after induction of diabetes by streptozocin.

Cell Preparation and Bioassays—Rat adipocytes were prepared from the fat pads of male Wistar rats (130–150 g) by collagenase digestion according to the method of Rodbell (37). Cell preparations showed more than 95% viability by Trypan blue exclusion at least 3 h after digestion. All bioassays were performed as described in figure legends. Glucose transport was carried out using 2-deoxy-D-[G - ^3H]glucose uptake (38),

and lipogenesis (the incorporation of U - ^{14}C -labeled glucose into lipids) was performed according to Moody *et al.* (39). Briefly, freshly prepared rat adipocytes were suspended in KRBH, 0.7% BSA buffer and divided into about 50 plastic vials. Each vial contained 0.5 ml of adipocyte suspension (about 1.5×10^6 cells). These were incubated for 2 h at 37°C under an atmosphere of 95% O_2 , 5% CO_2 with 0.16 mM [U - ^{14}C]glucose. Each assay contained vials with and without 17 nM insulin and the various test compounds. Lipogenesis was terminated by adding toluene-based scintillation fluid, and the extracted lipids were counted (39). Results are expressed as a percent of maximal insulin response. Only assays in which insulin activated lipogenesis 5–6-fold above basal (basal ~ 4000 cpm/ 1.5×10^6 cells/2 h, $V_{\text{insulin}} = 20,000$ – $24,000$ cpm/ 1.5×10^6 cells/2 h) were taken into consideration. Insulin activated lipogenesis in this assay at an ED_{50} value of 33 ± 3 pM. A concentration of 0.3 nM insulin and above already facilitated maximal (100%) response (*i.e.* Ref. 16). All assays were performed in duplicate or triplicate.

Western Immunoblot Analysis of GLUT4 in Subcellular Membranes Following Stimulation of Rat Adipocytes—Adipocytes prepared from 6-week-old rats were incubated with and without insulin and with L-Glu(γ)HXM alone and complexed with vanadate as specified in the figure. Cells were then homogenized and fractionated to low density microsomal membrane (LDM) and plasma membrane (PM) fractions by differential ultracentrifugation according to Ref. 40. Membrane proteins were then solubilized in sample buffer for 30 min at 25°C , resolved on 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and immunoblotted with anti-GLUT4 antisera (41). Visualization was performed by phosphorimaging. The relative intensity of bands corresponding to GLUT4 was quantitated using MacBas 1000.

^{51}V NMR Spectroscopy—The ^{51}V NMR spectra were recorded on a 200-MHz Bruker WPS4 (4.7T) spectrometer. Spectrum width of 16,000

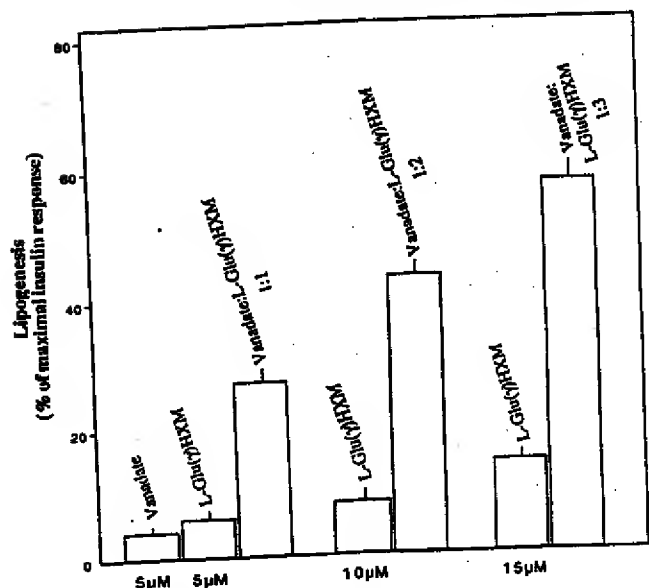


FIG. 2. Stimulation of lipogenesis at varying molar ratios of L-Glu(γ)HXM to vanadium(V). Freshly prepared rat adipocytes (3×10^5 cells/ml) suspended in KRB buffer, pH 7.4, containing 0.7% BSA were preincubated for 10 min with the indicated concentrations of 1:1 to 3:1 molar stoichiometry of L-Glu(γ)HXM to NaVO₃ or with free NaVO₃(V) and free L-Glu(γ)HXM. The cells were then supplemented with [¹⁴C]glucose, and lipogenesis was performed for 2 h at 37 °C. Radioactivity incorporated into extracted lipids was then determined. Maximal response (100%) is that obtained in the presence of 17 nM insulin.

H₂, a 90° pulse angle, and an accumulation time of 0.28 were used. The chemical shifts are reported relative to the external reference standard VOCl₂ (−490 ppm).

RESULTS

L-Glutamic Acid(γ)Monohydroxamate Potentiates Vanadium-evoked Lipogenesis in Rat Adipocytes—In this set of experiments, rat adipocytes were incubated for 10–20 min with submaximal concentrations of vanadate (10–30 μ M), L-Glu(γ)HXM (10–30 μ M), or an equimolar combination of them. The capacity to activate lipogenesis relative to insulin was then determined. As shown in Fig. 1, the combination was highly synergistic. For example, at 10 μ M vanadate or L-Glu(γ)HXM, lipogenesis was 17 ± 3 and $9 \pm 2\%$, respectively, whereas the combination produced a marked incredible $93 \pm 4\%$ activation of maximal insulin response. At 20 μ M, the extent of lipogenesis was 37 ± 3 , 20 ± 3 , and $121 \pm 7\%$, and at 30 μ M, it was 42 ± 4 , 23 ± 4 , and $143 \pm 7\%$ of maximal. Wortmannin (100 nM), an inhibitor of phosphatidylinositol 3-kinase, fully blocked the activating effects of vanadate, L-Glu(γ)HXM, and its combination with vanadate (Fig. 1, right columns). Thus L-Glu(γ)HXM potentiated vanadate-evoked lipogenesis about 3.5–5-fold; the higher concentrations reached a level that is about 140% of that achieved by saturating concentrations of insulin or vanadate. A finding of significant interest to us was the ability of L-Glu(γ)HXM to partially activate lipogenesis even in the absence of exogenous vanadium (Fig. 1). This finding is examined in great detail in connection with Fig. 6.

In Fig. 2, lipogenesis in rat adipocytes was evaluated at a fixed, low concentration of vanadate (5 μ M) with increasing concentrations of L-Glu(γ)HXM. Lipogenesis was negligible at 5 μ M vanadate or L-Glu(γ)HXM alone (4–6% of maximal insulin effect) but is augmented to $27.0 \pm 3\%$ when they were given in combination (at a molar stoichiometry of 1:1). At 2:1 and 3:1 Glu(γ)HXM-vanadium molar stoichiometry, lipogenesis expanded to 43 and 57%, respectively, of maximal response. Thus

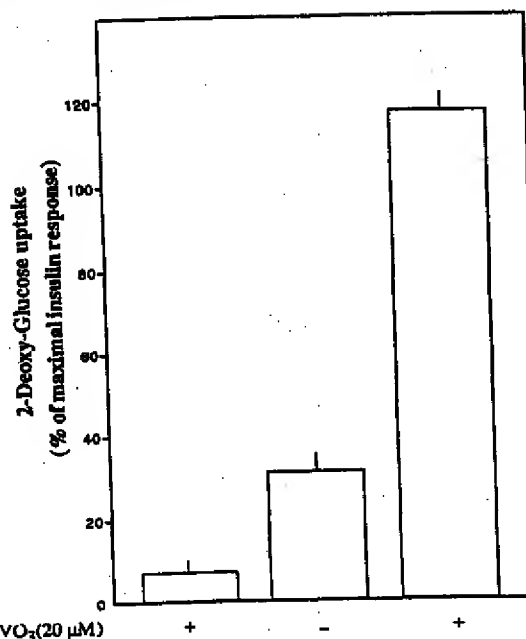


FIG. 3. Potentiation of hexose uptake by L-Glu(γ)HXM-vanadium (2:1). Adipocytes (2×10^5 cells/ml) suspended in KRBH buffer containing 1% BSA were preincubated in the presence and absence of insulin (17 nM), sodium metavanadate (20 μ M), L-Glu(γ)HXM (40 μ M), and their combination (at 1:2 molar stoichiometry). Aliquots (70 μ l) were transferred into tubes containing 2-deoxy-D-[6-³H]glucose (0.1 mM final concentration). Phloretin (0.1 mM) was added after 3 min for transport termination. This was followed by centrifugation of aliquots through a silicone layer.

a substantial synergistic effect is obtained at a 1:1 molar ratio and is increased further at a 2:1 molar stoichiometry and even higher, though much less pronounced, at a 3:1 molar ratio (Fig. 2).

L-Glu(γ)HXM Potentiates Vanadate-evoked Glucose Uptake—Fig. 3 shows activation of 2-deoxyglucose uptake by low concentrations of vanadate (20 μ M), L-Glu(γ)HXM (40 μ M), and by the 2:1 molar combination, of them. 2-Deoxyglucose undergoes insulin- or vanadate-evoked influx into the cell via the same transporters as glucose and is phosphorylated *in situ* to 2-deoxyglucose 6-phosphate with no further metabolism (42, 43). Therefore, this measurement reflects an effect on glucose entry into the cell in a manner largely independent of the metabolism of the endogenous saccharide. Vanadate (20 μ M) and L-Glu(γ)HXM (40 μ M) affected 2-deoxyglucose uptake of 7 ± 0.7 and $31 \pm 4\%$ of maximal insulin effect, respectively. Together they caused 2-deoxyglucose uptake $117 \pm 9\%$ of maximal insulin response (Fig. 3).

L-Glu(γ)HXM Alone and L-Glu(γ)HXM-Vanadate Lead to Translocation of GLUT4 from LDM to PM Fractions in Rat Adipocytes—Incubation of rat adipocytes with L-Glu(γ)HXM and L-Glu(γ)HXM-vanadate led to a decrease in the content of GLUT4 in the LDM fraction and an increase in the PM fraction (Fig. 4). The decrease in GLUT4 content in the low density lipoprotein fraction amounted to 32 ± 3 , 3 ± 1 , and $68 \pm 5\%$ of maximal insulin response upon incubating the cells with L-Glu(γ)HXM (40 μ M), vanadate (20 μ M, not shown), and the combination, respectively (calculated from Fig. 4). Under similar experimental conditions, L-Glu(γ)HXM, vanadate, and the combination activated 2-deoxyglucose uptake to an extent of 31 ± 4 , 7 ± 0.7 , and $117 \pm 9\%$ of maximal insulin response (Fig. 3), suggesting a contributing effect of the complex to glucose influx in addition to its effect in recruiting GLUT4 transporters

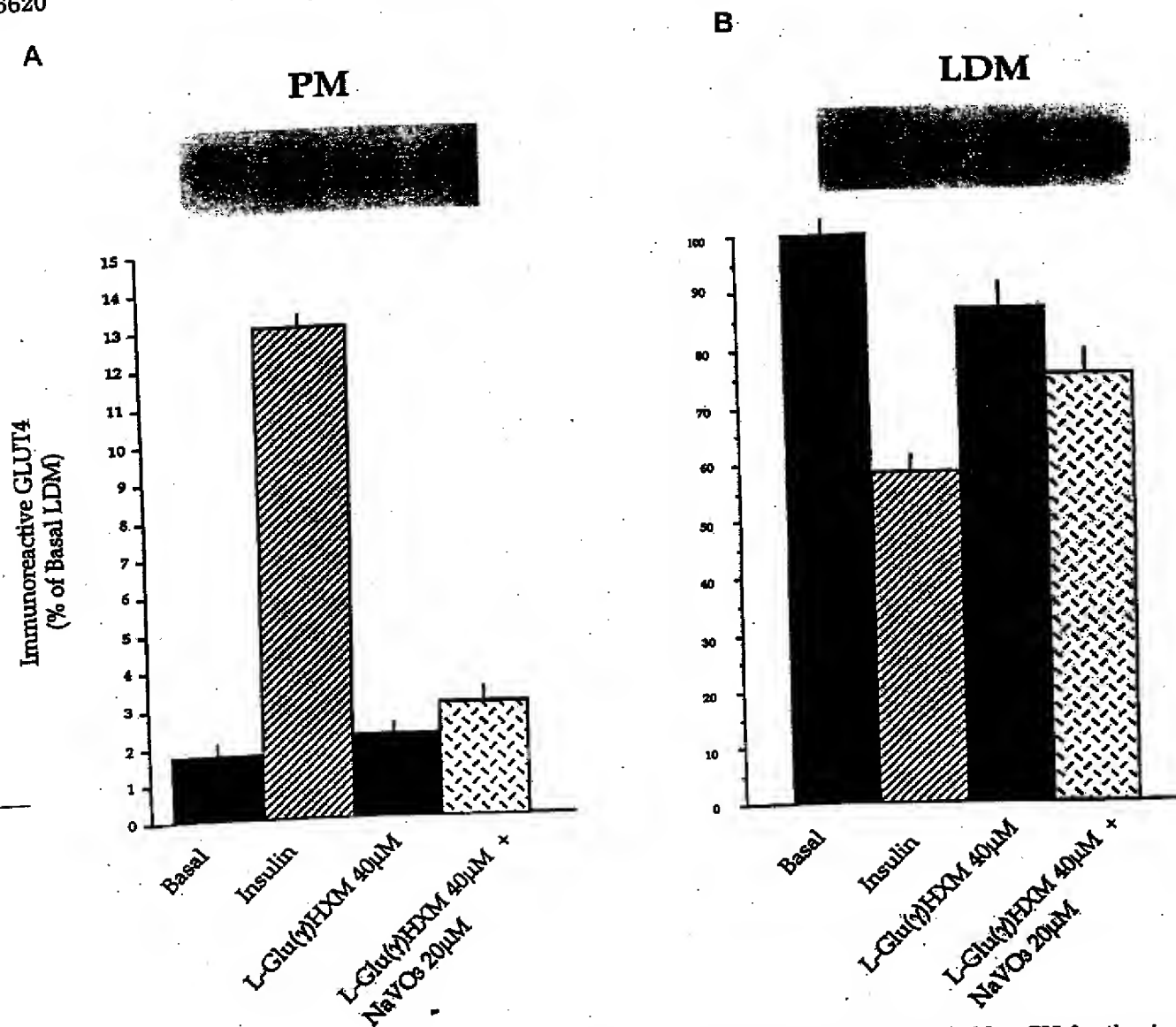


FIG. 4. L-Glu(γ)HXM alone or complexed with vanadate induces translocation of GLUT4 from LDM to PM fraction in rat adipocytes. Rat adipocytes were incubated for 30 min at 37 °C in the presence and the absence of insulin (17 nM) and the indicated concentrations of L-Glu(γ)HXM or L-Glu(γ)HXM-vanadate. Cells were then homogenized and fractionated to PM and LDM by differential ultracentrifugation, and GLUT4 protein was identified by Western immunoblot analysis ("Experimental Procedures"). Immunoreactive GLUT4 proteins were visualized by phosphorimaging (top panels) and were quantitated using MacBas 1000 software (histograms, bottom panels).

from the low density lipoprotein to the PM fraction.²

L-Glu(γ)HXM-Vanadate Normalizes Blood Glucose Levels in Streptozocin-treated Diabetic Rats—In the experiments summarized in Fig. 5, streptozocin-treated rats received intraperitoneally sodium metavanadate (0.05 mmol/kg body weight), L-Glu(γ)HXM (0.1 mmol/kg body weight), or a combination of the two compounds 8 days after the induction of diabetes. As shown in the figure, vanadate and L-Glu(γ)HXM, at these concentrations, had a rather minor effect in reducing the high circulating glucose levels characterizing these hyperglycemic rats. The combination, however, was highly efficient at normalizing blood glucose levels. Normoglycemia was evident 1 day after the first administration and remained so following two more administrations. The glucose levels then remained close

to normal for the next 8 days (Fig. 5).

Activation of Lipogenesis in Rat Adipocytes by L-Glu(γ)HXM in the Absence of Exogenous Vanadium—L-glutamic acid(γ)HXM also activated lipogenesis in the absence of added vanadium, and this effect was studied in detail (Fig. 6). The dose-response curve (Fig. 6A) indicates that activation is already evident at 5 μ M L-Glu(γ)HXM and that higher concentrations reach a level of $40 \pm 7\%$ of maximal insulin response (median effective dose = 35 ± 4 μ M). Other amino acid hydroxamates such as L-Tyr(α)HXM, Gly(α)HXM, and L-Ile(α)HXM also activated lipogenesis, but they were considerably less potent ($ED_{50} = 250 \pm 30$ μ M, $40 \pm 5\%$ of maximal insulin effect). L-Aspartic acid β -monohydroxamate showed higher lipogenic activity compared with the α -amino acid hydroxamates and was slightly less potent than L-Glu(γ)HXM ($ED_{50} = 45 \pm 7$ μ M, Fig. 6B). N-acetyl-L-Glu(γ)HXM and L-Glu(γ)HXM- α -methyl ester were virtually ineffective, indicating the need for a free α -amino and, to a somewhat lesser extent, a free

² I. Goldwasser, J. Li, E. Gershonov, M. Armoni, E. Karnieli, M. Fridkin, and Y. Shechter, manuscript in preparation.

α -carboxyl moiety for the activation of lipogenesis by L-Glu(γ)HXM in the rat adipose cell (Fig. 6C). Stereospecificity appears crucial as well, because the D-isomer of Glu(γ)HXM was ineffective. All these

findings indicate that activation of lipogenesis by L-Glu(γ)HXM depends on a specific entry of this L-amino acid analog into the adipose cell. Further investigation has led us to suggest that L-Glu(γ)HXM enters the adipose cell primarily through the non- Na^+ -dependent glutamine transport system.²

Several organic chelators, which potentiate the insulinomimetic activity of vanadium either *in vitro* or *in vivo*, have been documented. These include acetylacetonate (29), maltol (30, 31), picolinate (32, 33), and RL-252 (28). In Fig. 6D, we have examined whether they are capable of activating lipogenesis in the absence of exogenous vanadium. Unlike L-Glu(γ)HXM, none of these agents were able to activate lipogenesis in the rat adipose cell at concentrations of 100 μM (Fig. 6D) or lower (not shown).

Extensive Potentiation of L-Glu(γ)HXM-evoked Lipogenesis in Rat Adipocytes *In Vitro* Following Enrichment with Vanadium *In Vivo*—The findings presented in Figs. 1–4 have taught us that L-Glu(γ)HXM potentiates the insulinomimetic potency of vanadium and that activation of lipogenesis by L-Glu(γ)HXM alone never exceeds $40 \pm 7\%$ of maximal insulin effect (Fig. 6). To examine whether L-Glu(γ)HXM-evoked lipogenesis can be affected by the level of intracellular vanadium, a group of male Wistar rats received daily subcutaneous administrations of vanadate (0.1 mmol/kg/day) over a period of 5 days to raise the level of endogenous vanadium. Rats were then sacrificed 7 h after the last administration. Adipocytes were prepared, and the effect of L-Glu(γ)HXM on lipogenesis was compared with that in nontreated freshly prepared adipocytes. As shown in Fig. 7, vanadium-enriched adipocytes became dramatically sensitive to L-Glu(γ)HXM-evoked lipogenesis. This was valid both in terms of a leftward shift in the dose-response curve to L-Glu(γ)HXM ($\text{ED}_{50} = 6.4 \pm 0.3 \mu\text{M}$ versus $\text{ED}_{50} = 35 \pm 4 \mu\text{M}$ in control adipocytes) and in terms of the degree of lipogenesis (145 ± 15 versus $40 \pm 7\%$ of maximal insulin response, i.e. Fig. 6). At 10 μM , L-Glu(γ)HXM already stimulated lipogenesis and amounted to 120% of maximal insulin effect in the vanadium-enriched adipose cells (as opposed to only $8.0 \pm 1.5\%$ in control adipocytes) (Fig. 7).

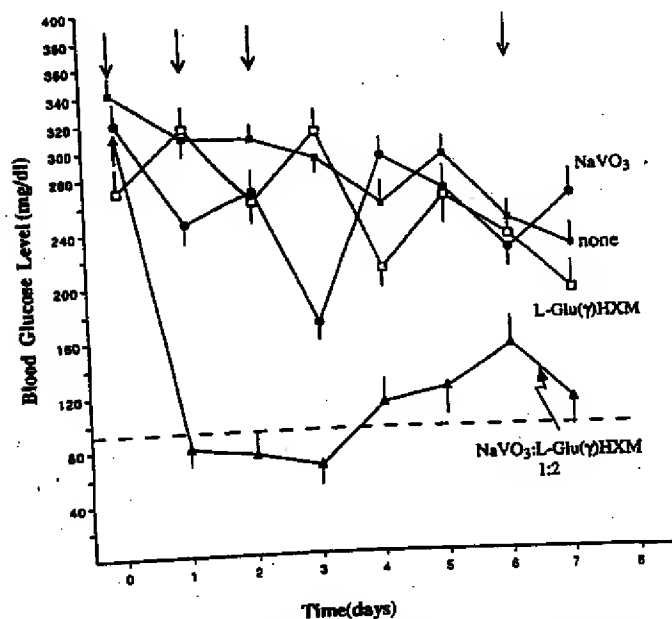


FIG. 5. Effect of L-Glu(γ)HXM-vanadate administration on blood glucose levels of streptozocin-treated rats. Male Wistar rats, 8 days after induction of diabetes (circulating glucose levels 310–340 mg/dalton), were divided into several groups. At the time points indicated by the arrows (intraperitoneal, at 11:00 a.m.), groups of diabetic rats received either vanadate (0.05 mmol/kg body weight \bullet), L-Glu(γ)HXM (0.1 mmol/kg body weight \square), L-Glu(γ)HXM (0.1 mmol/kg) and vanadate (0.05 mmol/kg, Δ), or none (\circ). Circulating glucose levels were determined daily (at 8:00 a.m.). Each point in the figure represents the arithmetic mean of plasma glucose for 5 rats. The dashed line indicates the arithmetic mean of plasma glucose of control healthy male Wistar rats.

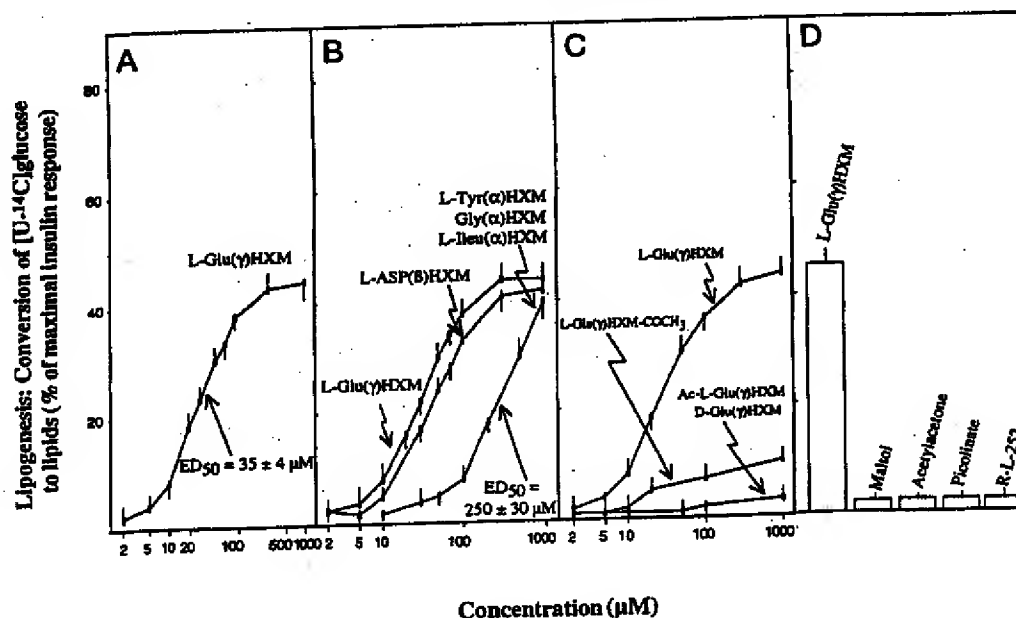


FIG. 6. Activation of lipogenesis by L-Glu(γ)HXM in the absence of exogenous vanadium. Comparison to other amino acid(α)hydroxamates and ineffectiveness of the D-isomer and of chemically modified L-Glu(γ)HXM derivatives. Freshly prepared adipocytes (3×10^5 cells/ml) suspended in KRB buffer, pH 7.4, containing 0.7% BSA were preincubated for 10 min with the indicated concentrations of the various test compounds. The cells were then supplemented with [^{14}C]glucose (final concentration 0.16 mM), and lipogenesis was performed for 2 h at 37 $^\circ\text{C}$. Radioactivity incorporated into extracted lipids was then determined. Maximal response (100%) is that obtained in the presence of 17 nM insulin.

FIG. 7. Activation of lipogenesis by L-Glu(γ)HXM. Comparison between normal adipocytes and vanadium-enriched adipocytes. Male Wistar rats received daily subcutaneously injected NaVO_3 (0.1 mmol/kg/day) for 5 days (called enriched vanadium rats). The rats were then sacrificed (7 h after the last administration). Lipogenesis was performed comparing the freshly prepared rat adipocytes (3×10^6 cells/ml) from nonenriched vanadium rats with the enriched ones suspended in KRB buffer, pH 7.4, containing 0.7% BSA. The cells were preincubated for 10 min with the indicated concentrations of L-Glu(γ)HXM. The cells were then supplemented with $[U-^{14}\text{C}]$ glucose, and lipogenesis was performed for 2 h at 37 °C. Radioactivity incorporated into extracted lipids was then determined. Maximal response (100%) is that obtained in the presence of 17 nM insulin.

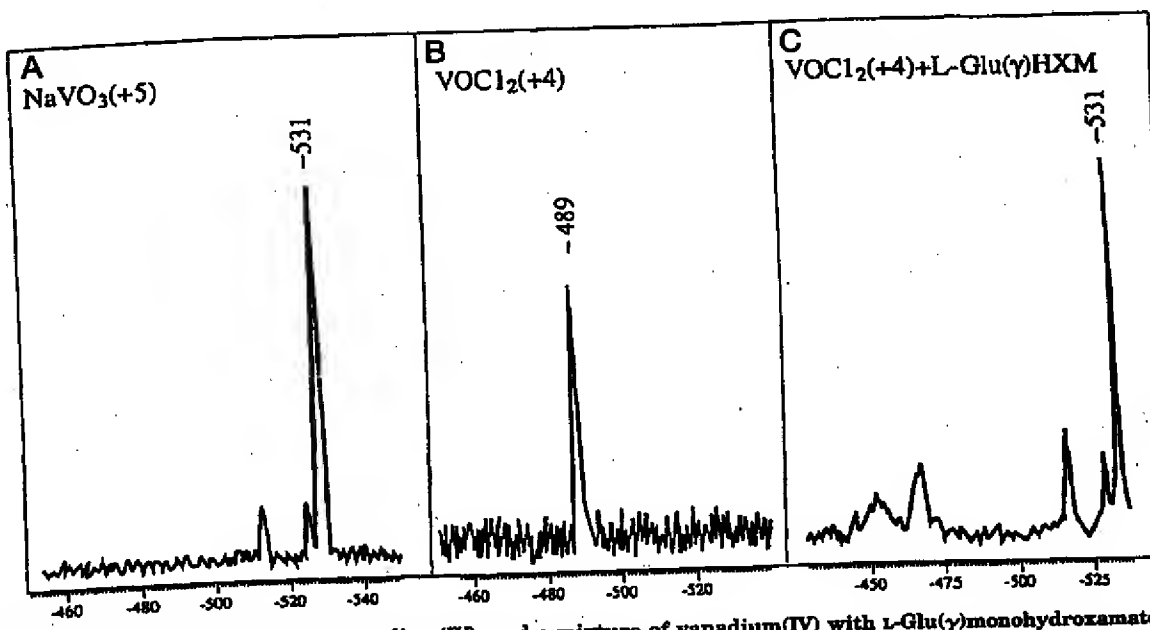
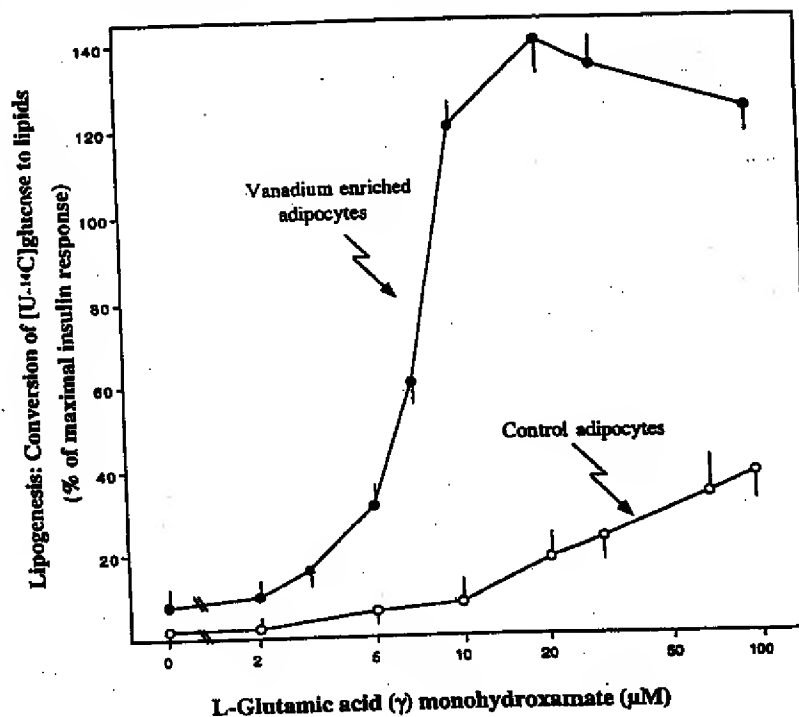


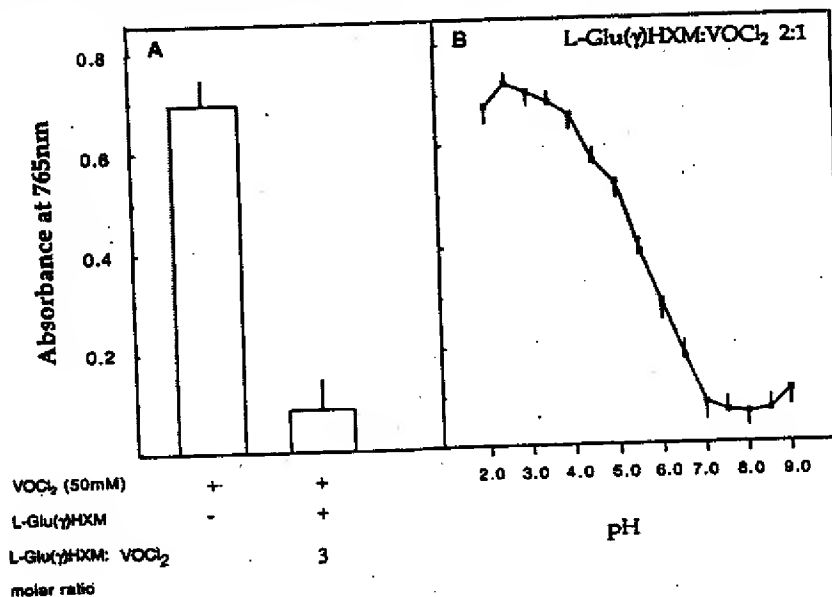
FIG. 8. ^{51}V NMR spectra of vanadium(V), vanadium(IV), and a mixture of vanadium(IV) with L-Glu(γ)monohydroxamate. A, ^{51}V NMR spectrum of sodium metavanadate (20 mM, pH 7.1); B, ^{51}V NMR spectrum of vanadium dichloride(IV) (20 mM, pH 6.8); C, ^{51}V NMR spectrum of a mixture (1:1 molar ratio) of VOCl_2 (IV) and L-Glu(γ)HXM (20 mM, pH 7.0). Spectra were monitored with fresh solutions. In C, the spectrum was monitored 5 min after the addition of L-Glu(γ)HXM to VOCl_2 .

Spectroscopic Studies—Previously we found in cell-free experiments that vanadium(IV), at neutral pH values, undergoes slow spontaneous oxidation to vanadium(V). This occurs similarly in the presence of 10 mM reduced glutathione, an ineffectual reductant of vanadium(V), at neutral pH values with a $t_{1/2}$ value of 1 ± 0.1 h at 25 °C (29). The results summarized in Fig. 8 show the ^{51}V NMR spectra of vanadium dichloride(IV) at pH 7.0 prior to and after the addition of L-Glu(γ)HXM. Vanadium dichloride(IV) appeared as a single peak with a chemical shift of -490 ppm in its ^{51}V spectrum, indicating one main species present at >95% purity. Upon the addition of L-Glu(γ)HXM (1

equivalent), the chemical shift of vanadium(IV) at -490 ppm disappeared within minutes and the principal chemical shift characterizing vanadium(V) at -530 ppm appeared (Fig. 8).

Vanadium(IV) (i.e. vanadyl sulphate or VOCl_2) has a characteristic "blue" absorbance with $\epsilon_{765 \text{ nm}} = 14 \pm 0.3$, whereas vanadium(V) does not absorb at all at this wavelength (29). The addition of 2–3 equivalents of L-Glu(γ)HXM to VOCl_2 (IV) (50 mM at pH 7.5) led rapidly to a near total decrease in vanadium(IV) absorbance at 765 nm (Fig. 9). Fig. 8B depicts complex formation as a function of the pH in the range of pH 2–9. Decrease is minimal at pH 4.0, quite significant at pH 5.0,

FIG. 9. Decrease in absorbance of vanadium(IV) at 765 nm upon addition of L-Glu(γ)HXM. Effect of pH: A, left column, absorbance of VOCl_2 alone (50 mM in H_2O); right column, absorbance of VOCl_2 (50 mM) and L-Glu(γ)HXM (150 mM) titrated with NaHCO_3 to pH 7.4. B, samples of VOCl_2 (50 mM) and L-Glu(γ)HXM (100 mM) in H_2O were titrated either with HCl or with NaHCO_3 before absorbance at 765 nm and were monitored to obtain the pH values indicated in the figure. L-Glu(γ)HXM alone does not absorb at 765 nm. Vanadium dichloride alone, which tends to precipitate at neutral pH values, remains completely soluble at all pH values in the presence of two or more equivalents of L-Glu(γ)HXM.



half-maximal at pH 5.7, and reaches a stable plateau at pH range 7–9 (Fig. 9B).

DISCUSSION

It has been consistently observed that chelated vanadium compounds are more potent than the free metaloxide in facilitating the metabolic actions of insulin. This was demonstrated *in vitro* with systems like rat adipocytes, as well as in diabetic rodents such as streptozocin-treated hyperglycemic rats (28–33, 44). Because of the variations in the experimental models used, the oxidation state of vanadium applied, and the different administration modes, the basis for the higher insulinomimetic potencies of complexed vanadium remained rather speculative. Because this topic has immediate therapeutic relevance, we looked for new vanadium chelators characterized by: (a) higher synergistic potencies than previously documented for vanadium chelators with respect to vanadium-evoked glucose uptake and glucose metabolism both *in vitro* and in diabetic rats *in vivo*, (b) low indices of toxicity, and (c) reasonable solubility in aqueous, neutral media after complexation with vanadium.

In this study, we have introduced the L-isomer of glutamic acid(γ)monohydroxamate as it satisfactorily fulfilled the above criteria. It potentiated vanadium-activated hexose uptake, glucose metabolism, and recruitment of GLUT4 transporters from LDM to PM fractions (Figs. 1–4). *In vivo* it potentiated the efficacy of vanadium to lower blood glucose levels in streptozocin rats (Fig. 5). This amino acid analog has negligible toxicity in mammals.² Both L-Glu(γ)HXM alone and its complexes with vanadium are fairly soluble in aqueous media at neutral pH values. An important finding was that L-Glu(γ)HXM alone, in the absence of exogenous vanadium, showed a reasonable amount of insulinomimetic activity in that it activated glucose uptake and glucose metabolism in the rat adipose cell (Figs. 1–3). Further investigation revealed that this activating effect is unique to the L-isomer of Glu(γ)HXM but is not facilitated by the D-isomer. Nonmodified α -amino and α -carboxyl moieties appear essential. This intrinsic activity is exclusive to L-Glu(γ)HXM not being shared by any of the other vanadium chelators that potentiate the actions of vanadium *in vivo* or *in vitro* (Fig. 6, A–D, and Refs. 28–33). Our assumption that L-Glu(γ)HXM permeates into the cell interior and transforms the “dormant” intracellular vanadium pool into an insulinomimetic-activated species gains credence from the dramatic sen-

sitization of vanadium-enriched adipocytes to L-Glu(γ)HXM-evoked lipogenesis (Fig. 7).

It should be mentioned at this point that because of the extreme complexity of aqueous vanadium chemistry (reviewed in Refs. 46–49), the intracellular milieu of the mammalian cell is still “a black box” with respect to the state and the form of entered vanadium. With the endogenously present vanadium pool, experiments have shown that it exists mostly as vanadium(IV), though some researchers may wonder even about this experimental finding because vanadium in its IV oxidation state is only stable at acidic pH values (pH < 3.0) and readily oxidizes to vanadium(V) at neutral pH even in the presence of high glutathione concentrations (28, 46). The intracellular vanadium pool, however, can be preserved in its IV oxidation form at neutral pH values if it is chelated by ascorbic acid (not shown) or to endogenous proteins (50, 51). At the low physiological level of intracellular vanadium, the cell should have the capacity to chelate all the endogenous vanadium.

Our experimental findings that L-Glu(γ)HXM alone enhances glucose uptake and glucose metabolism (Figs. 1 and 2) together with the apparent rapid conversion of vanadium(IV) to vanadium(V) upon complexation (Figs. 8 and 9) strongly support the contention that vanadium(V) rather than vanadium(IV), and in a chelated form, is the active insulinomimetic species that facilitates the activation of glucose uptake and its metabolism in rat adipocytes. Although most of our previous cell-free experiments support this conclusion, we were not fully convinced prior to the completion of this study. This is because protein phosphotyrosine phosphatases (with *p*-nitrophenylphosphate as a substrate) are inhibited by both vanadium(IV) and vanadium(V), free or chelated, at nearly the same concentrations (see Ref. 52). On the other hand, adipose non-receptor protein-tyrosine kinases, whether cytosolic or membranal, are with one exception activated by vanadium(V) but not at all by vanadium(IV) (22, 23). We have only observed vanadium(IV)-evoked activation of nonreceptor protein-tyrosine kinases when membranal protein phosphotyrosine phosphatases were extracted with Triton X-100 and added to the cytosolic protein-tyrosine kinase fraction (29). These experimental conditions, however, are not likely to occur in the intact cell system. For example, broken plasma membrane fragments (or deoxycholate-treated membranal fragments) did not sup-

port activation of cytosolic protein-tyrosine kinases in the presence of vanadium(IV) (29).

In summary, L-Glu(γ)HXM appears superior to previously documented organic chelators of vanadium in potentiating its activation of glucose uptake and glucose metabolism *in vitro* and *in vivo*. Taken together with earlier studies, this may be attributed to one or more of the following: (a) increased efficiency of this specific combination to permeate into cells or tissues; (b) a favorable 5-coordinated, rather than octahedral topography of this complex in an aqueous, neutral environment (Ref. 50);² and/or (c) higher intracellular stability of the L-Glu(γ)HXM-vanadium complex. Finally, we have recently observed that vanadate does not inhibit alkaline phosphatase in the presence of L-Glu(γ)HXM.² This inhibitory effect of vanadate (53) is undesirable from our point of view as it may contribute to vanadium toxicity in mammals, but not to the efficacy of vanadium to manifest the metabolic actions of insulin (reviewed in Ref. 54). This and other basic and diabetological aspects raised here are being further investigated.

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Evidence That Glucose Metabolism Regulates Leptin Secretion from Cultured Rat Adipocytes*

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ABSTRACT

Circulating leptin secreted from adipocytes is correlated with fat mass and plasma insulin concentrations in humans and rodents. Plasma leptin, insulin, and glucose decrease during fasting and increase after refeeding; however, the underlying mechanisms regulating the changes of leptin secretion are not known. To investigate the role of insulin-stimulated glucose metabolism in the regulation of leptin secretion, we examined the effects of insulin and inhibitors of glucose transport and metabolism on leptin secretion from rat adipocytes in primary culture. Insulin (0.16–16 nM) increased leptin secretion over 96 h; however, the increase in leptin was more closely related to the amount of glucose taken up by the adipocytes ($r = 0.64$; $P < 0.0001$) than to the insulin concentration *per se* ($r = 0.20$; $P < 0.28$), suggesting a role for glucose transport and/or metabolism in regulating leptin secretion.

2-Deoxy-D-glucose (2-DG), a competitive inhibitor of glucose transport and phosphorylation, caused a concentration-dependent (2–50 mg/dl) inhibition of leptin release in the presence of 1.6 nM insulin. The inhibitory effect of 2-DG was reversed by high concentrations of

glucose. Two other inhibitors of glucose transport, phloretin (0.05–0.25 mM) and cytochalasin-B (0.5–50 μ M), also inhibited leptin secretion. Inhibition of leptin secretion by these agents was proportional to the inhibition of glucose uptake ($r = 0.60$ to 0.86 ; all $P < 0.01$). Two inhibitors of glycolysis, iodoacetate (0.005–1.0 mM) and sodium fluoride (0.1–5 mM), produced concentration-dependent inhibition of leptin secretion in the presence of 1.6 nM insulin. In addition, both 2-DG and sodium fluoride markedly decreased the leptin (*ob*) messenger RNA content of cultured adipocytes, but did not affect 18S ribosomal RNA content.

We conclude that glucose transport and metabolism are important factors in the regulation of leptin expression and secretion and that the effect of insulin to increase adipocyte glucose utilization is likely to contribute to insulin-stimulated leptin secretion. Thus, *in vivo*, decreased adipose glucose metabolism may be one mechanism by which fasting decreases circulating leptin, whereas increased adipose glucose metabolism would increase leptin after refeeding. (*Endocrinology* 139: 551–558, 1998)

THE ADIPOCYTE hormone, leptin is implicated in the regulation of food intake, energy expenditure, and body fat stores (1). Circulating leptin decreases after fasting or caloric restriction in both humans (2–4) and rodents (5–7), and increases a number of hours after refeeding (3, 6). In humans, there is a nocturnal rise of plasma leptin (8), which has been hypothesized to be due to a delayed effect of insulin released during earlier meals. Consistent with this hypothesis, insulin increases expression of the *ob* gene in rodents (9–11) and in adipocytes *in vitro* (12, 13) after a number of hours. In humans, plasma insulin and leptin concentrations decrease in parallel after weight loss, independently of changes of adiposity (14). Furthermore, plasma leptin is negatively correlated with insulin sensitivity independently of adiposity in subjects with impaired glucose tolerance (15).

Short term insulin administration does not affect plasma leptin concentrations in human subjects (16, 17), but increases in circulating leptin have been reported after 4–6 h of high dose insulin administration (18, 19). These studies by

necessity require the infusion of large amounts of glucose to prevent hypoglycemia. Similarly, prolonged hyperglycemia in response to extended glucose infusions increases plasma leptin after several hours in nonhuman primates (20) and human subjects (21); however, glucose administration also markedly increases endogenous insulin levels. Therefore, the role of insulin *per se* on the adipocyte vs. the effect of insulin to increase glucose flux into adipocytes was not addressed by these experiments.

Several lines of evidence have led us to hypothesize that glucose is an important regulator of leptin expression and secretion. First, increases in *ob* messenger RNA (mRNA) after glucose administration in mice are more closely related to plasma glucose concentrations than to plasma insulin concentrations (22). Second, infusion of small amounts of glucose to prevent the decline of glycemia during fasting in humans also prevents the decrease in plasma leptin (2). Third, the decrease in plasma leptin during marked caloric restriction in humans is better correlated with the decrease in plasma glucose than with changes in insulinemia (4). Fourth, we have found that low plasma leptin levels in streptozotocin diabetic rats are acutely increased by insulin administration in proportion to the degree of glucose lowering (23). Lastly, lowering plasma glucose concentrations in hyperglycemic insulin-dependent diabetic human subjects by

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infusing insulin at rates that produced physiological insulinemia increases circulating leptin (24).

To investigate the mechanisms by which glucose influences leptin secretion, we adapted and modified an *in vitro* system for culturing rat adipocytes in which the adipocytes are anchored in a defined mixture of extracellular matrix components (25). This matrix, Matrigel, appears to simulate normal basement membrane attachment of cells and may allow cell to cell interactions between adipocytes. Cells cultured in this system are, therefore, in an environment closer to their normal physiological milieu than in systems where adipocytes are free floating in the culture medium. Adipocytes cultured on Matrigel have been shown to maintain many of their differentiated characteristics and, in contrast with free-floating adipocytes, show no sign of dedifferentiation after 6 days of culture (25, 26). With this system we have investigated the regulation of leptin secretion by glucose and insulin and the effects of inhibitors of adipocyte glucose transport and metabolism on leptin secretion. The leptin (*ob*) mRNA content of the adipocytes after culture with insulin and inhibitors was also examined.

Materials and Methods

Materials

DMEM and FBS were purchased from Life Technologies (Grand Island, NY). The media were supplemented with 6 ml each of MEM nonessential amino acids, penicillin/streptomycin (5000 U/ml/5000 µg/ml), and nystatin (10,000 U/ml; all from Life Technologies) per 500 ml DMEM. BSA fraction V, HEPES, collagenase (*Clostridium histolyticum*; type II, Sigma Chemical Co., St. Louis, MO; SA, 456 U/mg), insulin, D-glucose, sodium fluoride (NaF), phloretin, iodoacetate, and fructose were purchased from Sigma Chemical Co. Matrigel matrix was purchased from Becton Dickinson (Franklin Lakes, NJ). 2-Deoxy-D-glucose (2-DG) was obtained from U.S. Biochemical Corp. (Cleveland, OH). Six-well Falcon tissue culture plates were purchased from Fisher Scientific (Pittsburgh, PA). Nylon filters were purchased from Tetko (Kansas City, MO).

Animals

Male Sprague-Dawley rats were obtained from Charles River (Wilmington, MA). Animals were housed in hanging wire cages in temperature controlled rooms (22–24°C) with a 12-h light-dark cycle and fed Purina chow diet (Ralston-Purina, St. Louis, MO) and given deionized water *ad libitum*. The study protocol was approved by the University of California-Davis animal care and use committee.

Cell isolation/preparation

Adipocytes were prepared from epididymal fat pads of male Sprague-Dawley rats (300–600 g) anesthetized with halothane. Epididymal fat depots were resected under aseptic conditions, and adipocytes were isolated by collagenase digestion according to the Rodbell procedure (27) with minor modifications as described below. The fat pads were minced into pieces in Krebs-Ringer HEPES buffer (pH 7.4; containing 5 mM D-glucose, 2% BSA, 135 mM NaCl, 2.2 mM CaCl₂·2H₂O, 1.25 mM MgSO₄·7H₂O, 0.45 mM KH₂PO₄, 2.17 mM Na₂HPO₄, and 10 mM HEPES). Adipose tissue fragments were digested in the same buffer in the presence of type II collagenase (25 mg/2 ml buffer/g tissue) at 37°C with gentle shaking at 60 cycles/min for 45 min. The resulting cell suspension was diluted in 24 ml cold HEPES-phosphate buffer. Isolated adipocytes were separated from undigested tissue by filtration through a 400-µm nylon mesh and washed three times. For washing, cells were centrifuged at 500 rpm for 5 min. Each time the supernatant was discarded, and the adipocytes were resuspended in Krebs-Ringer HEPES buffer, with the final wash being in 0, 5, or 10 mM glucose culture medium supplemented

with 1% or 5% FBS. The isolated adipocytes were then incubated for 30 min at 37°C before being plated in Matrigel-coated culture plates.

Adipocyte culture

Matrigel was thawed on ice to a liquid and uniformly applied to the surface of the culture dish (300 µl Matrigel/35-mm well). After the incubation, 150 µl of the adipocyte suspension (2:1 ratio of packed cells to medium) were plated on the liquid matrix. The warmth of the cells and buffer caused the Matrigel to gel around the adipocytes, effectively anchoring them to the culture dish. After a 30-min incubation at 37°C, 2 ml warm culture medium supplemented with FBS were added. The cells were maintained in an incubator at 37°C in 6% CO₂ for 96 h.

The initial medium concentration of glucose for the cultures conducted in the insulin dose-response experiment was 10.0–10.5 mM (180–190 mg/dl) to ensure that the cells would not deplete the glucose supply during the 96-h incubation when higher concentrations of insulin were used. Only 1% FBS was used in the insulin dose-response study to minimize the small amount of insulin present in the serum, which at 1% was less than 0.1 µU/ml. In the fructose study, medium made with glucose-free DMEM and 1% fetal serum was used to minimize the amount of glucose available to the adipocytes (<0.1 mmol/liter). However, it was not possible to eliminate all glucose from culture preparation because the Matrigel matrix itself contains ~4.2 mmol/liter glucose. For the fructose experiment, the Matrigel was diluted 1:2 with glucose-free medium to approximately 1.5 mmol/liter glucose.

In the other experiments with inhibitors of glucose transport, 2-DG (28), phloretin (29), and cytochalasin B (30), or with inhibitors of glycolysis, iodoacetate (31), and NaF (32), the initial medium glucose concentration was (5.0–5.5 mM; 90–100 mg/dl) with 5% fetal serum. These agents were used at concentrations at or below those typically employed to inhibit glucose transport or glycolysis in adipocytes (28–32). Cytochalasin B was initially dissolved in ethanol and diluted to 0.5% ethanol in the well with the highest dose. Therefore, the medium in all wells in the cytochalasin B experiment was equalized to 0.5% ethanol. Aliquots of adipocytes from each animal were divided into wells with the responses to insulin, the various inhibitors, or fructose being compared with those of an appropriate control well containing adipocytes from the same animal. In a preliminary insulin dose-response study, we found that medium leptin concentrations in the presence of insulin were not increased over those in control medium (no insulin) until after 24 h of incubation. Therefore, for the remainder of the studies, 300-µl samples (15% of the medium volume) were collected at 24, 48, 72, and 96 h and replaced with 300 µl fresh medium containing the appropriate concentrations of glucose, insulin, and/or inhibitors. Cultures were observed daily with a phase contrast microscope. After 96 h, a subset of the culture plates was frozen until analyzed for leptin (*ob*) mRNA content by Northern blot.

Assays

Leptin concentrations in the medium were determined with a sensitive and specific RIA for mouse leptin as previously described (7) (Linco Research, St. Charles, MO). Leptin concentrations in medium from cultured rat adipocytes measured with this assay are very similar to those obtained with a newly developed assay specific for rat leptin. With the rat-specific assay, measured leptin concentrations in culture medium were $86 \pm 3\%$ of the mouse values and were highly correlated between the two assays ($r = 0.97$; $P < 0.0001$; unpublished data). Therefore, measurements of rat leptin made with the mouse assay provide a reliable measurement of leptin concentrations. The intra- and interassay coefficients of variation for this assay are 4.0% and 11.2%, respectively (7). The antibody used in the assay does not cross-react with insulin, proinsulin, glucagon, pancreatic polypeptide or somatostatin. Glucose and lactate were measured with a YSI glucose analyzer (model 2300, Yellow Springs Instruments, Yellow Springs, OH).

Northern blot procedure

The following procedures were performed on culture plates incubated with 5 mM glucose and 5% fetal serum alone (control), 1.6 nM insulin, and 1.6 nM insulin with 10 mg/dl 2-DG or 1 mM NaF for 48 and 96 h. Northern blot analysis was performed as previously described (33).

In brief, 1 ml RNazol B (Tel-Test, Friendswood, TX) was added directly to the wells containing the adipocytes and matrix. The solution was repetitively taken in and expelled from the pipette to maximize dissolution of the adipose tissue. UV absorbance and integrity gels were used to estimate RNA. To allow loading of equal mass of RNA in each well, after analysis of leptin mRNA using a single-stranded complementary DNA probe followed by quantification of bands on a phosphorimager as well as from film, the blots were reanalyzed using a probe complementary to mouse 18S ribosomal RNA. Leptin mRNA was then normalized with respect to the 18S ribosomal signal, according to the absolute signal. The 18S RNA results were virtually identical in all cases. In particular, experimental conditions did not influence the 18S ribosomal signal.

Calculations and data analysis

The uptake of glucose was assessed by measuring the concentration of glucose in the medium in each well before and after 96 h of incubation and calculating the decrease over 96 h. To examine the relationship between adipocyte carbon flux and leptin secretion in response to increased insulin-mediated glucose uptake, the amount of carbon released as lactate per amount of carbon taken up as glucose over 96 h was calculated as $\Delta[\text{lactate}]/\Delta[\text{glucose}]$, where Δ is the change, and expressed as a percentage. The area under the curve for leptin concentrations in the medium between 0–96 h was calculated by the trapezoidal method. The means of two groups were compared by paired *t* test. The means of more than two groups were compared by ANOVA. To examine the relationships between the medium concentrations of insulin or inhibitors employed, the amount of glucose taken up by the adipocytes, and leptin secretion, simple and multiple linear regression analyses were performed with a statistics software package (StatView for Macintosh, Abacus Concepts, Inc., Berkeley, CA). Data are expressed as the mean \pm SEM.

Results

Responses to insulin (0.16–16.0 nM)

The effects of insulin on leptin secretion, and the relationship between glucose uptake by adipocytes cultured with different concentrations of insulin and leptin secretion were examined. Insulin produced a concentration-dependent increase in glucose uptake by the cultured adipocytes ($r = 0.61$; $P < 0.0002$ vs. insulin concentration), as assessed by the decrease in glucose in the medium (Fig. 1A). With no added insulin, the medium glucose concentration decreased from 10.1 ± 0.1 to 8.2 ± 0.3 mmol/liter (Δ , -1.9 ± 0.3 mmol/liter; $P < 0.0001$). The addition of 0.16, 1.6, and 16.0 nM insulin increased glucose uptake (Δ glucose, -2.7 ± 0.4 , -3.3 ± 0.3 , and -3.9 ± 0.4 mmol/liter, respectively; all $P < 0.01$ vs. no insulin). Insulin also produced a concentration-dependent increase in lactate production ($r = 0.70$; $P < 0.0001$), which was well correlated with the decrease in glucose in the medium over 96 h ($r = 0.61$; $P < 0.0002$), suggesting that a significant portion of the glucose entering the adipocytes was metabolized only as far as lactate and released from the cells into the medium (34, 35).

Leptin secretion was increased over the control value by all three concentrations of insulin (Fig. 1B). The production of lactate was not related to the leptin response ($r = 0.10$; $P = 0.59$). The area under the leptin concentration curve (AUC) from 0–96 h was independently related to the decrease in glucose in the medium during the incubation (Fig. 1C), but not to the insulin concentration (Table 1). Similarly, with a multiple regression model, the AUC for leptin was related to the decrease in glucose, but not to the insulin concentration. In addition, the percentage of carbon released as lactate per amount of carbon taken up as glucose was calculated. Over-

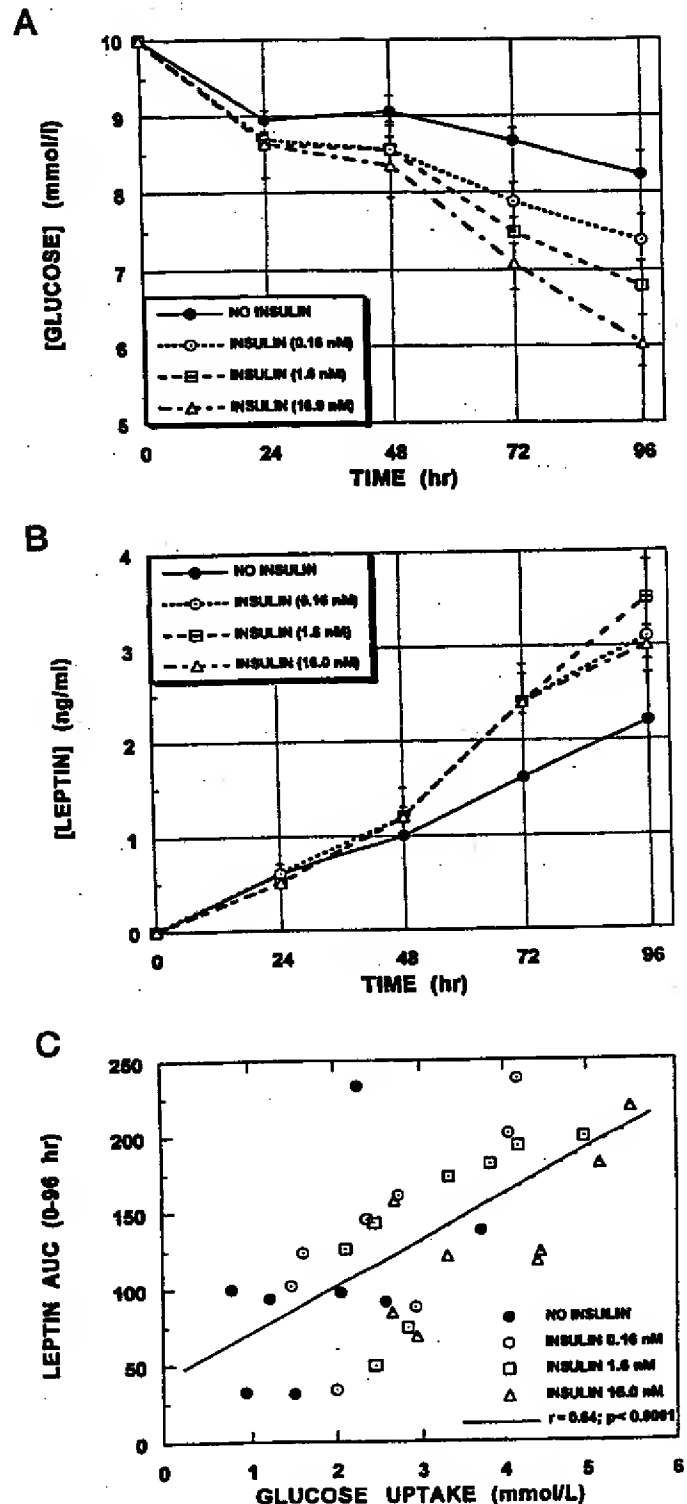


FIG. 1. A, Glucose concentrations in medium from 0–96 h from isolated rat adipocytes in primary culture with insulin concentrations from 0–16 nM ($n = 8$ /treatment). B, Leptin concentrations from 0–96 h from isolated rat adipocytes in primary culture with insulin concentrations from 0–16 nM ($n = 8$ /treatment). C, Relationship between glucose uptake, as assessed by the decrease in glucose in the culture medium, and leptin secretion, expressed as the AUC from 0–96 h, during incubation of adipocytes with 0–16 nM insulin ($n = 32$).

TABLE 1. Relationship between glucose uptake (Δ Gluc), leptin secretion (Δ Lept) and the concentration of insulin or inhibitors of glucose uptake and metabolism after 96-h incubation of adipocytes with insulin and insulin plus inhibitors (2-DG, phloretin, cytochalasin-B, iodoacetate, or sodium fluoride at varying concentrations; see *Materials and Methods*)

Insulin or inhibitor (n)	Simple Regression				Multiple regression	
	Δ Lept vs. Δ Gluc		Δ Lept vs. [inhibitor]		Δ Lept vs. Δ Gluc	Δ Lept vs. [inhibitor]
	r	P	r	P	(P)	(P)
Insulin (32)	0.64	0.0001	0.20	0.28	0.0001	0.09
2-DG (38)	0.67	0.0001	0.51	0.001	0.001	0.29
Phloretin (38)	0.86	0.0001	0.78	0.0001	0.0012	0.75
Cytochalasin-B (19)	0.60	0.01	0.58	0.02	0.22	0.25
Iodoacetate (34)	0.83	0.0001	0.74	0.0001	0.0001	0.17
Sodium fluoride (28)	0.85	0.0001	0.60	0.001	0.0001	0.73

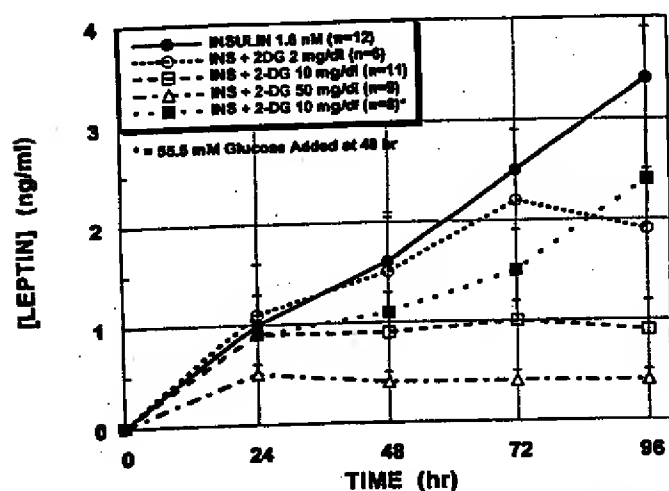


FIG. 2. Effects of inhibiting glucose transport and metabolism with 2-DG on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin and the effect of adding glucose (55 mM) at 48 h on the inhibition of leptin secretion produced by 10 mg/dl 2-DG.

all, in the insulin experiment between 10–68% of the amount of carbon taken up as glucose was released as lactate (mean, $34 \pm 2\%$). There was no direct relationship between this parameter and the insulin concentration; however, it was inversely proportional to the amount of leptin secreted, as expressed by the 0–96 h leptin AUC ($r = 0.64$; $P < 0.0001$). By multiple regression analysis, the relationship between glucose conversion to lactate and leptin secretion was not significantly related to lactate production ($P = 0.06$), but leptin secretion was equally related to both the change in glucose and the amount of glucose carbon released as lactate (both $P < 0.001$).

Effects of 2-DG (2–50 mg/dl)

The effect of inhibiting glucose uptake and metabolism with 2-DG on leptin secretion and its relationship to adipocyte glucose uptake were examined. 2-DG at a concentration of 50 mg/dl completely inhibited glucose uptake (Δ , 0.1 ± 0.3 mmol/liter) in the presence of 1.6 nM insulin (Δ glucose, -4.0 ± 0.6 mmol/liter) and inhibited the leptin response (AUC 0–96 h) by $69 \pm 4\%$ ($P < 0.0001$) compared with insulin alone (Fig. 2A). At a lower concentration of 2-DG (10 mg/dl), glucose uptake was still markedly inhibited (Δ , -0.1 ± 0.4

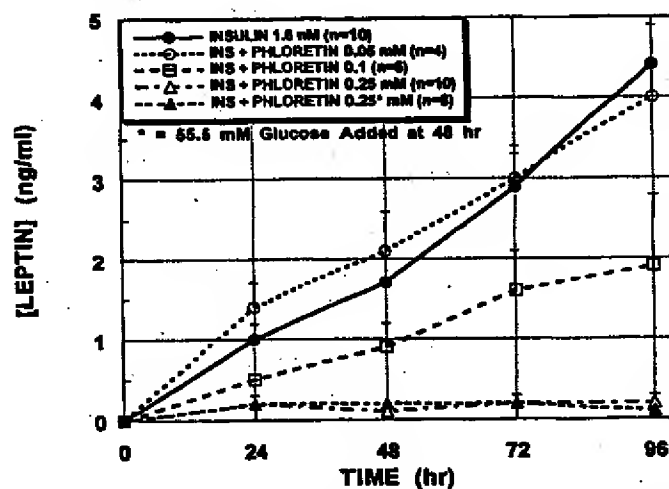


FIG. 3. Effects of inhibiting glucose transport with phloretin on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin and the effect of adding glucose (55 mM) at 48 h on the inhibition of leptin secretion produced by 0.25 mM phloretin.

mmol/liter) and leptin secretion was inhibited by $47 \pm 5\%$ ($P < 0.0001$). The lowest concentration of 2-DG (2 mg/dl) produced less of an inhibition of glucose uptake (Δ , -1.5 ± 0.9 mmol/liter; $P < 0.01$ vs. insulin alone). At this concentration, the leptin response was not significantly inhibited until the 96 h point ($P < 0.02$ vs. insulin alone; Fig. 2A).

Overall, the change in leptin at 96 h was related to the concentration of 2-DG and was well correlated with the decrease in medium glucose (Table 1). By multiple regression, the leptin concentration in the medium at 96 h was significantly correlated with the change in glucose, but not to the 2-DG concentration (Table 1). The addition of glucose (55.5 mM) at 48 h reversed the inhibition of leptin secretion produced by 2-DG at 10 mg/dl by 96 h ($P < 0.01$ vs. 2-DG; NS vs. insulin alone; Fig. 2).

Effects of phloretin (0.05–0.25 mM)

The effect of inhibiting glucose uptake with phloretin on leptin secretion was examined. Phloretin at a concentration of 0.25 mM completely inhibited leptin secretion (Fig. 3). The 0–96 h AUC for leptin was inhibited by $91 \pm 2\%$ of insulin alone ($P < 0.0001$). This higher concentration of phloretin (0.25 mM) also completely blocked glucose uptake in the

presence of 1.6 nM insulin (Δ glucose, 0.7 ± 0.1 mmol/liter). Overall, the leptin response was inversely related to the concentration of phloretin and was highly correlated with the decrease in glucose in the medium (Table 1). However, by multiple regression, the leptin response was correlated with the decrease in glucose, but not with the concentration of phloretin (Table 1). The addition of 55.5 mM glucose at 48 h did not reverse the inhibition of leptin secretion by phloretin (Fig. 3).

Effects of cytochalasin B

The effect of inhibiting glucose uptake with cytochalasin B on leptin secretion was examined. Cytochalasin B produced a concentration-dependent inhibition of glucose uptake and leptin secretion (Fig. 4). The leptin response was significantly correlated with glucose uptake by simple regression (Table 1), but was not significantly correlated with glucose uptake (as observed with the other inhibitors; Table 1) by multiple regression, perhaps due to the smaller number of replicates ($n = 19$) in this experiment.

Effects of iodoacetate (0.005–1.0 mM)

The effect of inhibiting glycolysis with iodoacetate on leptin secretion was examined. Iodoacetate at 1.0, 0.1, and 0.01 mM markedly inhibited glucose uptake (Δ glucose, -0.1 ± 1.1 , 0.5 ± 0.2 , and 0.3 ± 0.2 mmol/liter, respectively) and leptin secretion. The 0–96 h AUC for leptin was inhibited by $-95 \pm 2\%$, $-91 \pm 2\%$, and $-87 \pm 3\%$, respectively, compared with insulin alone; (all $P < 0.0001$). The lowest concentration of iodoacetate (0.005 mM) produced less of an inhibition of glucose uptake (Δ glucose, -1.8 ± 0.8 mmol/liter) and less of an inhibition of leptin secretion ($-51.0 \pm 16\%$) than insulin alone ($P < 0.02$; Fig. 5). By simple regression, the release of leptin was related to the concentration of iodoacetate and was highly correlated with the change in glucose in the medium (Table 1). However, by multiple regression, the leptin secreted at 96 h was related to the change in glucose, but not to the concentration of iodoacetate (Table 1).

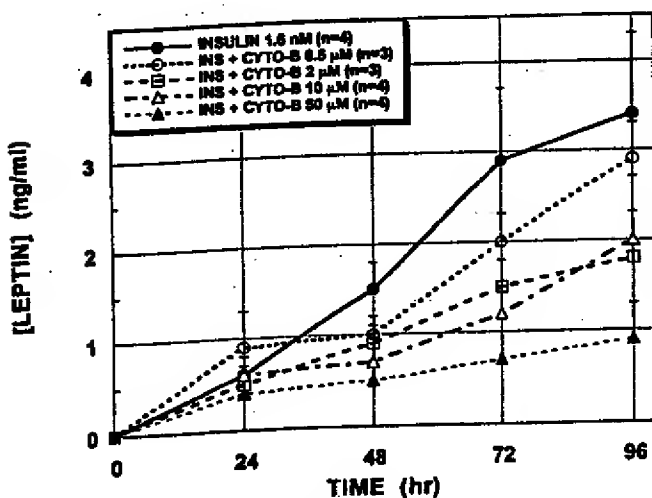


FIG. 4. Effects of inhibiting glucose transport with cytochalasin B on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin.

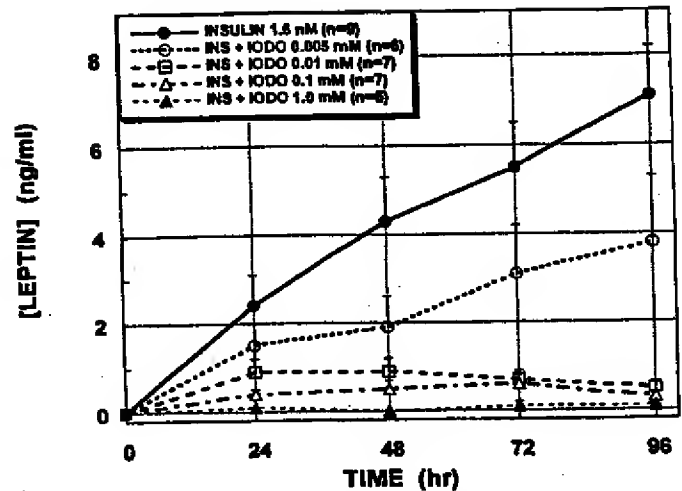


FIG. 5. Effects of inhibiting glycolysis with iodoacetate on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin.

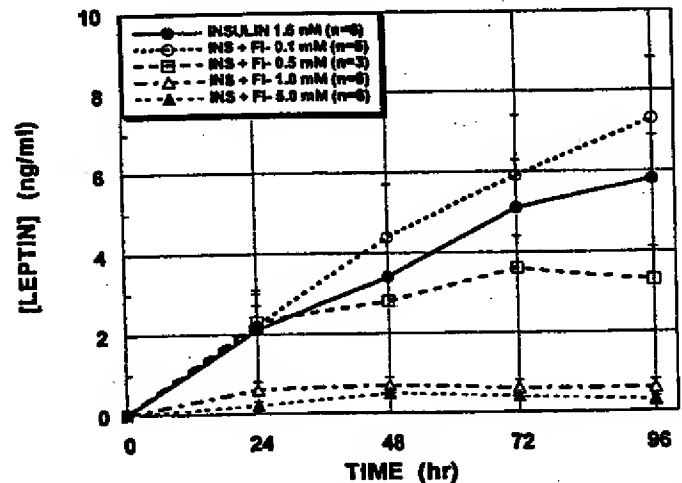


FIG. 6. Effects of inhibiting glycolysis with NaFl on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin.

Effects of NaFl (0.1–5.0 mM)

The effect of inhibiting glycolysis with NaFl was examined. The two highest concentrations of NaFl (5.0 and 1.0 mM) completely inhibited glucose uptake (Δ glucose, 0.2 ± 0.1 and 0.0 ± 0.3 mmol/liter, respectively). The 0.5 mM concentration of NaFl produced less of an inhibition of glucose uptake (Δ , -2.1 ± 0.6 mmol/liter), and the lowest concentration (0.1 mM) of NaFl did not inhibit glucose uptake (Δ glucose, -3.9 ± 0.5 mmol/liter) compared with the effect of insulin alone. The two highest concentrations of NaFl (5.0 and 1.0 mM) markedly inhibited leptin secretion ($-81 \pm 6\%$ vs. insulin alone; $P < 0.0001$). The next concentration of NaFl (0.5 mM) produced an intermediate inhibition of leptin secretion ($-47 \pm 15\%$ of insulin alone; $P < 0.05$). The 0.1-mM concentration of NaFl did not inhibit leptin secretion ($-4 \pm 15\%$ vs. insulin alone; $P = \text{NS}$; Fig. 6).

Overall, the decline in medium glucose was significantly

correlated with the concentration of NaFl and highly correlated with the 96 h leptin concentration (Table 1). By multiple regression, the amount of leptin secreted at 96 h was strongly correlated with the change in glucose in the medium ($P < 0.0001$), but not to the NaFl concentration (Table 1).

Effects of insulin, 2-DG, and NaFl on leptin (*ob*) mRNA and 18S ribosomal RNA

The effects of inhibiting glucose uptake and metabolism with 2-DG or NaFl on leptin gene expression and ribosomal 18S RNA were examined. As shown in Fig. 7A, leptin (*ob*)

mRNA was detectable in adipocytes incubated for 48 h either with 1.6 nM insulin or without insulin (control). However, the leptin mRNA signal was reduced to near undetectable levels when adipocytes were incubated with 1.6 nM insulin and either 2-DG (10 mg/ml) or 1.0 mM NaFl (Fig. 7A). The effect of 2-DG and NaFl was specific, because in the same samples there was no effect of these concentrations of 2-DG or NaFl on 18S ribosomal RNA (Fig. 7B) or on nonspecific RNA bands (with a different mol wt than leptin mRNA) that could be detected on the Northern blots after long exposures (data not shown). Leptin mRNA was significantly reduced by 2-DG or NaFl regardless of whether the signal was normalized for 18S ribosomal signal ($P = 0.0174$). Qualitatively similar effects of 2-DG or NaFl were observed in cultures incubated for 96 h ($P = 0.0228$; data not shown).

Effects of fructose (5 mM)

The addition of 5 mM fructose to medium of cultures in which the glucose concentration was minimized by diluting the Matrigel 1:2 and using glucose-free DMEM with 1% serum augmented leptin secretion after 48 h. The initial response in the control wells was probably due to the residual glucose (~1.5 mmol/L) in the diluted Matrigel. However, both the integrated AUC from 0–96 h ($P < 0.02$) and the leptin concentration at 96 h ($P < 0.01$) were increased by fructose (Fig. 8).

Discussion

In the present study we found that addition of physiological concentrations of insulin stimulates leptin secretion from isolated rat adipocytes in primary culture. In this *in vitro* system we did not see an acute effect of insulin on leptin secretion. This is in agreement with previous reports that have demonstrated that the expression of *ob* gene and leptin protein release are not acutely regulated by insulin *in vivo* and *in vitro* (17, 36). The strong correlation between adipocyte glucose uptake measured by the decrease in glucose in the media during incubation with insulin and the amount of

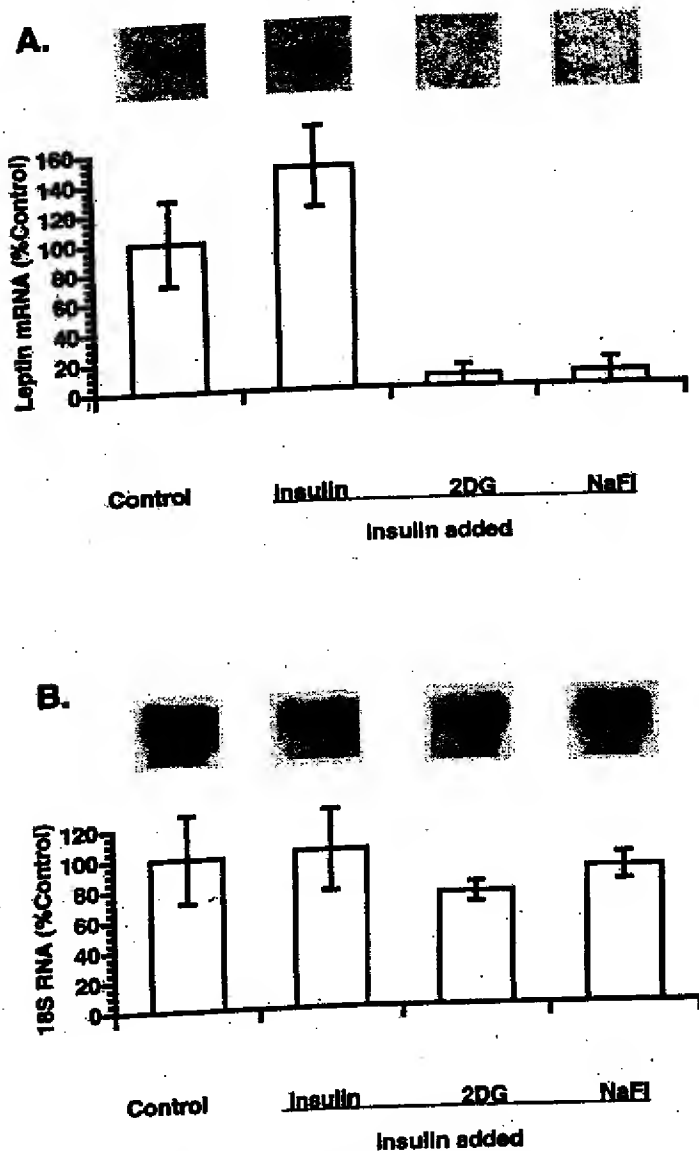


FIG. 7. A, Effects of no insulin (control), 1.6 nM insulin, and 1.6 nM insulin plus 10 mg/dl 2-DG or 1.0 mM NaFl on leptin (*ob*) mRNA after 48 h of incubation, as assessed by Northern blots. The inset above each bar is representative of the signal obtained for each condition. B, Effects of control (no insulin), 1.6 nM insulin, and 1.6 nM insulin plus 10 mg/dl 2-DG or 1.0 mM NaFl on 18S ribosomal RNA after 48 h of incubation, as assessed by Northern blots. The inset above each bar is representative of the signal obtained for each condition.

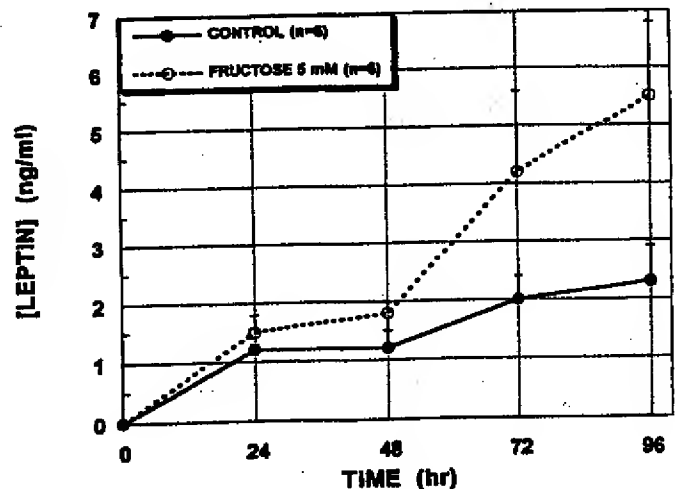


FIG. 8. Effect of fructose (5 mM) on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture with a low (~1.5 mmol/liter) initial glucose concentration ($n = 6/\text{treatment}$).

leptin secreted from isolated adipocytes is consistent with the hypothesis that the rate of glucose metabolism is a determinant of leptin secretion. In addition, the absolute insulin concentration was not related to the leptin response, independently of the effect of insulin to increase glucose uptake.

Blockade of glucose transport with 2-DG, phloretin, or cytochalasin B at concentrations at or below those typically used in adipocytes (29, 30) produced a dose-dependent decrease in leptin secretion in the presence of high physiological concentrations of insulin. The competitive inhibition produced by 2-DG could be reversed by the addition of a high concentration of glucose, suggesting that 2-DG did not inhibit leptin secretion via a nonspecific toxic effect on the adipocytes. As expected, the inhibition by phloretin was not reversed by glucose, as phloretin is not a competitive inhibitor and, therefore, produces an irreversible inhibition of glucose transport that is not readily overcome by high glucose concentrations. These experiments provide evidence that glucose uptake is required to increase leptin secretion from isolated adipocytes despite the presence of high physiological insulin concentrations.

Inhibition of glycolysis with either iodoacetate or NaFl at low concentrations (31, 32) also produced concentration-dependent inhibition of leptin secretion in the presence of insulin. When glycolysis is inhibited, glycolytic intermediates accumulate, resulting in a secondary impairment of glucose uptake. As with primary blockade of glucose uptake, during inhibition of glucose metabolism by either glycolytic inhibitor, the amount of glucose taken up over 96 h of incubation was highly correlated with the amount of leptin secreted despite the presence of insulin. These results suggest that the stimulation of leptin secretion by insulin is unlikely to be due to a direct effect of insulin *per se*, but is secondary to the effect of insulin to stimulate glucose uptake and metabolism in adipocytes.

We also found that inhibition of glucose transport and metabolism with 2-DG or glycolysis with NaFl markedly inhibited leptin (*ob*) gene expression, as assessed by Northern blot analysis of leptin mRNA. In the same cultures, 18S ribosomal RNA levels were unaffected by either 2-DG or NaFl, suggesting that the decrease in leptin gene expression was not due to a nonspecific overall effect of these inhibitors to impair adipocyte RNA synthesis. In addition, we examined the amount of heparin-released lipoprotein lipase (LPL) from adipocytes cultured with the various inhibitors (data not presented). Although LPL was modestly decreased by the inhibitors (~25–50% of insulin-stimulated levels), the suppression of leptin secretion was significantly greater (80–90%), suggesting a relative specificity of blocking glucose uptake and metabolism on leptin secretion *vs.* that on another protein (LPL) produced by adipose tissue. Lastly, the effects of the blockers to inhibit leptin expression and secretion are unlikely to be due to a depletion of adipocyte energy stores, as it is known that adipocytes can generate energy (ATP) by oxidizing fatty acids via mitochondrial β -oxidation (37, 38).

Taken together, these data suggest a physiological role for glucose in the regulation of leptin expression and secretion by adipocytes. Accordingly, we hypothesize that during fasting, when circulating insulin and glucose concentrations are low and glucocorticoids are elevated, leptin secretion de-

clines secondary to decreased glucose transport into adipose tissue. Upon refeeding, increases in circulating insulin and glucose and the resulting increases in adipose glucose uptake and metabolism stimulate leptin secretion and restores circulating leptin concentrations to prefasting levels. This model, therefore, can explain the effects of fasting and refeeding on circulating leptin in humans (2–4) and rodents (5–7). In addition, the nocturnal increase in plasma leptin observed in humans could potentially arise as a delayed consequence of increased insulin-stimulated glucose metabolism following meals (8). The effect of glucose infusions to prevent the fall of plasma leptin during fasting in human subjects may be similarly mediated (2).

Thus, leptin secretion appears to reflect the amount of glucose transported and metabolized by adipose tissue. There is convincing evidence that suggests that a significant portion of glucose entering adipose tissue is metabolized to lactate and released (34, 35). This lactate may contribute to the pool of gluconeogenic precursors during fasting. Our results show that when a smaller proportion of glucose carbon taken up by adipocytes is released as lactate, more leptin is secreted. These data are consistent with the changes in leptin secretion observed during fasting and refeeding. In addition, fructose, in the presence of low glucose concentrations, stimulates leptin secretion, demonstrating that a non-glucose substrate can induce the adipocyte to secrete leptin and suggesting that stimulation of leptin secretion by glucose metabolism occurs downstream of phosphofructokinase.

In summary, blockade of glucose transport or inhibition of glycolysis inhibits leptin secretion from and gene expression in isolated cultured adipocytes. The secretion of leptin is directly proportional to the amount of glucose taken up by the adipocytes. These results suggest that leptin secretion is linked to glucose transport and metabolism and help to explain the known effects of feeding/fasting and long term glucose and insulin administration on circulating leptin concentrations.

Acknowledgments

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Effects of Metformin and Vanadium on Leptin Secretion from Cultured Rat Adipocytes

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Abstract

MUELLER, WENDY M., KIMBER L. STANHOPE, FRANCINE GREGOIRE, JOSEPH L. EVANS, AND PETER J. HAVEL. Effects of metformin and vanadium on leptin secretion from cultured rat adipocytes. *Obes Res.* 2000;8:530–539.

Objective: We have reported that glucose utilization regulates leptin expression and secretion from isolated rat adipocytes. In this study, we employed two antidiabetic agents that act to increase glucose uptake by peripheral tissues, metformin and vanadium, as pharmacological tools to examine the effects of altering glucose utilization on leptin secretion in primary cultures of rat adipocytes.

Research Methods and Procedures: Isolated adipocytes (100 μ L of packed cells per well) were anchored in a defined matrix of basement membrane components (Matrigel) with media containing 5.5 mM glucose and incubated for 96 hours with metformin or vanadium. Leptin secretion, glucose utilization, and lactate production were assessed.

Results: Metformin (0.5 and 1.0 mM) increased glucose uptake in the presence of 0.16 nM insulin by $37 \pm 10\%$ ($p < 0.005$) and $62 \pm 8\%$ ($p < 0.0001$) over insulin alone, respectively. Metformin from 0.5 to 5.0 mM increased lactate production by $105 \pm 43\%$ ($p < 0.025$) to $202 \pm 52\%$ ($p < 0.0025$) and at 1.0 and 5.0 mM increased the proportional rate of glucose conversion to lactate by $78 \pm 18\%$ ($p < 0.005$) and $166 \pm 41\%$ ($p < 0.0025$), respectively. At concentrations less than 0.5 mM, metformin did not affect leptin secretion, but at 0.5 mM, the only concentration that significantly increased glucose utilization without increasing glucose conversion to lactate, leptin secretion was modestly stimulated (by $20 \pm 9\%$; $p < 0.05$). Concentrations from 1.0 to 25 mM inhibited leptin secretion by $25 \pm 8\%$

($p < 0.005$) to $89 \pm 4\%$ ($p < 0.0001$). Across metformin doses, leptin secretion was inversely related to the percentage of glucose taken up and released as lactate ($r = -0.74$; $p < 0.0001$). Vanadium (5 to 20 μ M) increased glucose uptake from $20 \pm 7\%$ ($p < 0.01$) to $34 \pm 13\%$ ($p < 0.02$) and increased lactate production at 5 μ M by $17 \pm 8\%$ ($p < 0.025$) and 10 μ M by $61 \pm 20\%$ ($p < 0.02$) but did not alter the conversion of glucose to lactate. Vanadium (5 to 50 μ M) inhibited leptin secretion by $33 \pm 6\%$ ($p < 0.0025$) to $61 \pm 8\%$ ($p < 0.0001$).

Discussion: Both metformin and vanadium increase glucose uptake and inhibit leptin secretion from cultured adipocytes. The inhibition of leptin secretion by metformin is related to an increase in the metabolism of glucose to lactate. The inhibition by vanadium most likely involves direct effects on cellular phosphatases. We hypothesize that the effect of glucose utilization to stimulate leptin production involves the metabolism of glucose to a fate other than anaerobic lactate production, possibly oxidation or lipogenesis.

Key words: glucose uptake, lactate production, anaerobic metabolism

Introduction

The adipocyte hormone, leptin, has a central role in the regulation of food intake, energy expenditure, and body fat stores (1,2). Circulating leptin concentrations are well correlated with adipose stores in humans (3–5) and animals (5–7). However, leptin production is also acutely regulated by nutritional status. For example, circulating leptin decreases after fasting (6,8–10) or energy restriction (11,12) and increases after refeeding or overfeeding (9,13). These changes of circulating leptin are disproportionate to the relatively small changes of body fat. Although the regulation of leptin expression and secretion is incompletely understood, changes of insulin secretion during fasting and refeeding precede changes of circulating leptin concentrations. There is a growing body of evidence that suggests a role for insulin and glucose in mediating changes of circulating leptin levels in vivo. For example, infusion of small amounts of glucose to prevent the reductions of insulin and

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glucose during fasting in humans also prevents the decrease in plasma leptin (8). Although insulin administration does not acutely increase plasma leptin concentrations in human subjects (14,15) increases have been reported after 4 to 6 hours during insulin infusions producing supraphysiological (16,17) or physiological (18) increments of plasma insulin levels. Similarly, prolonged hyperglycemia and hyperinsulinemia in response to extended glucose infusions increases plasma leptin after several hours in nonhuman primates (7) and human subjects (19). Lastly, leptin concentrations increase 4 to 6 hours after high carbohydrate meals, which induce large plasma insulin and glucose responses in humans (20).

In vitro studies have shown that insulin increases leptin expression and secretion in isolated rodent (21–23) and human (15,24) adipocytes. It has not, however, been clear whether the effect of insulin to increase leptin production is a direct consequence of increased insulin signaling or might be indirectly mediated by insulin's actions on glucose metabolism. Several in vivo studies have provided support for the latter explanation. First, glucose administration induces increases of *ob* mRNA expression, which are more closely related to changes of plasma glucose than to plasma insulin concentrations (25,26).

In addition, the decrease in plasma leptin during marked caloric restriction in humans is better correlated with the decrease in plasma glucose than with changes in insulinemia (11,12). Furthermore, we have found that low plasma leptin levels in insulin-deficient streptozotocin diabetic rats are acutely increased by insulin administration in proportion to the degree of glucose lowering (27). Further support from in vitro experiments for a role for adipose glucose utilization in the regulation of leptin production is provided by our recent report that increased glucose metabolism is an important mediator of insulin-stimulated leptin expression and secretion. Blockade of glucose uptake or inhibition of glycolysis decreases *ob* gene expression and leptin secretion in isolated rat adipocytes (28). However, glucose uptake, by itself, only seems to be important in that glucose must first be taken up by the adipocytes before it can be metabolized. Rather than glucose uptake per se, the inverse relationship observed, between the proportional conversion of glucose to lactate and leptin secretion by isolated adipocytes (28), suggests that a regulatory step for glucose metabolism to mediate changes of leptin production involved the metabolism of glucose to a point beyond the anaerobic metabolism of glucose-derived pyruvate to lactate.

Metformin and vanadium are two antidiabetic agents, which are able to enhance glucose uptake and utilization by peripheral tissues (29,30). In the present study, we employed metformin and vanadium as pharmacological tools to examine the effects of altering adipocyte glucose utilization on leptin production in primary cultures of isolated adipocytes. Glucose utilization, lactate production, and lep-

tin secretion were measured over 96 hours in isolated rat adipocytes cultured in a basement membrane matrix that maintains adipocyte differentiation.

Research Methods and Procedures

Materials

Media (Dulbecco's modified Eagle's medium [DMEM]) and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY). The media was supplemented with 6 mL each of minimal essential medium amino acids, penicillin/streptomycin (5000 U/mL/5000 µg/mL), and nystatin (10,000 U/mL; all from Life Technologies) per 500 mL of DMEM. Bovine serum albumin fraction V, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES), collagenase (*Clostridium histolyticum*, type II; specific activity, 456 U/mg), insulin, and metformin were purchased from Sigma Chemical Co. (St. Louis, MO). Matrigel matrix was purchased from Becton Dickinson (Franklin Lakes, NJ). Bis(maltolato) oxovanadium(IV) (BMOV), an organified form of vanadium (31), was a gift from Drs. John McNeill and Violet Yuen, Department of Pharmaceutical Sciences, University of British Columbia, Vancouver, BC, Canada. Six-well Falcon plates were purchased from Fisher Scientific (Pittsburgh, PA). Nylon filters were purchased from Tetko (Kansas City, MO).

Animals

Male Sprague-Dawley rats (3 to 6 months of age) were obtained from Charles River (Wilmington, MA). Animals were housed in hanging wire cages in temperature-controlled rooms (22 °C) with a 12-hour light-dark cycle and fed Purina chow diet (Ralston-Purina, St. Louis, MO) and given deionized water ad libitum. Animal use and care was in accordance with the National Institutes of Health Guide for the Use and Care of Laboratory Animals and conducted in facilities accredited by the American Association for Accreditation of Laboratory Animal Care. The study protocol was approved by the Administrative Animal Use and Care Committee at University of California, Davis.

Methods

Cell Isolation/Preparation. Adipocytes were prepared from epididymal fat pads from male Sprague-Dawley rats weighing 300 to 600 g. Epididymal fat depots were resected from halothane-anesthetized rats under aseptic conditions, and adipocytes were isolated by collagenase digestion by the Rodbell method (32) with minor modifications as previously described (28). The isolated adipocytes were then incubated for 30 minutes at 37 °C before being plated and cultured on Matrigel-coated plates.

Adipocyte Culture. Adipocytes were maintained in culture anchored to a basement membrane matrix (Matrigel, Becton Dickinson). Although all in vitro systems have

inherent advantages and disadvantages, advantages of this system compared with cultures containing free-floating adipocytes are that the matrix simulates their normal basement membrane attachment and that the cells are maintained in close proximity to each other, allowing direct cell-to-cell contact. Together the cell contact and basement membrane attachment help to maintain differentiation, because adipocytes have a strong tendency to dedifferentiate in long term (>24-hour) culture. In addition, the matrix and the small amount of serum in the media both contain growth factors, which are also likely to help maintain cell differentiation. Furthermore, the adipocytes in this system are not exposed to toxic levels of oxygen at the interface of the media and the incubator atmosphere, as opposed to free-floating adipocytes which aggregate at the surface of the media. An advantage of the system over those containing minced adipose tissue is that all of the cells in the culture are equally exposed to the nutrients and the oxygen dissolved in the media. Thus, although clearly different from the *in vivo* situation, we believe that this system provides a more physiological environment than most systems for maintaining adipocytes in long term culture. In the case of the present studies, the goal was to examine the direct actions of metformin and vanadium on leptin production and adipocyte metabolism. Therefore, the advantage of employing *in vitro* experimentation for this purpose over *in vivo* models was that it was possible to control confounding variables, such as effects of these agents on food intake, which would indirectly influence leptin production via changes of insulin secretion (18,20). Unlike an *in vivo* system, in this study the environment surrounding the adipocytes within the individual wells of each culture plate was identical with the exception of the presence or absence and the concentration of metformin or vanadium, allowing assessment of the direct effects of the treatments.

In culturing each suspension, Matrigel was first thawed on ice to a liquid and uniformly applied to the surface of culture dishes (300 μ L of Matrigel/35-mm well). After the incubation period, 150 μ L of the adipocyte suspension (2:1 ratio of packed cells to media) were plated on the liquid Matrigel matrix. Adipocytes from each suspension were thoroughly mixed with a transfer pipette before plating to insure that a similar number of adipocytes with a similar size distribution were added to the control and experimental wells for each suspension. The warmth of the added cells and media caused the Matrigel to gel around the adipocytes, effectively anchoring them to the culture dish. After a 30-minute incubation at 37 $^{\circ}$ C, 2 mL of warm culture medium was added. The cells were maintained in an incubator at 37 $^{\circ}$ C for 96 hours with 6% CO₂. Aliquots of adipocytes from each animal were divided into wells, with the

different concentrations of either metformin or vanadium (as detailed below). In each plate an appropriate control well contained adipocytes from the same animal. Adipocytes were incubated with media (DMEM) containing 5.5 mM (100 mg/dL) glucose plus 5% FBS at five concentrations of Metformin (0.1, 0.25, 0.5, 1.0, 5.0, and 25.0 mM). A low basal concentration of insulin (0.16 nM) was added to the incubations performed with metformin, because metformin is thought to act in part by increasing insulin signaling (33,34). Vanadium was added at four concentrations (5.0, 10.0, 20.0, and 50.0 μ M) in DMEM with 5.0 to 5.5 mM glucose and 1% FBS. Adipocytes were cultured with vanadium without added insulin, because vanadium action is considered to be largely independent of insulin (35,36). To examine the responses to insulin in the adipocytes obtained from each adipocyte suspension in the vanadium experiments, a separate well containing 1.6 nM insulin was included for each suspension. In all experiments, aliquots of media (300 μ L, 15% of the media volume) were collected from culture wells and replaced with fresh media containing the appropriate concentrations of metformin or vanadium at 24, 48, 72, and 96 hours.

Assays. Leptin concentrations in the medium were determined with a sensitive and specific radioimmunoassay for rat leptin (37) with reagents obtained from Linco Research, St. Charles, MO. Glucose and lactate were measured with a glucose analyzer (model 2300, YSI, Yellow Springs, OH).

Data Analysis. The uptake of glucose was assessed by measuring the concentration of glucose in the media in each well before and at 24, 48, 72, and 96 hours of incubation and calculating the decrease over 96 hours after correcting for the amount of glucose that was removed during each 24-hour media sampling and the amount added by the replacement of fresh media (15% of total volume). Lactate production was calculated as the increase of media lactate at 24, 48, 72, and 96 hours by correcting for the amount of lactate removed by sampling and added with media replacement. To examine the relationship between adipocyte carbon flux and leptin secretion in adipocytes cultured with metformin or vanadium, the amount of carbon released as lactate per amount of carbon taken up as glucose over 96 hours was calculated as lactate production/glucose utilization and expressed as a percentage (28). Cumulative leptin production was calculated as the change of media leptin concentrations at 24, 28, 72, and 96 hours with correction for the amount of leptin removed during sampling. The area under the curve for leptin production between 0 and 96 hours was calculated by the trapezoidal method. The experimental results from each adipocyte suspension prepared from a single animal were analyzed in relation to a control well from the same suspension. Given the

individual variation in leptin responses between animals and/or suspensions, it was not appropriate to compare means from control and treated adipocytes from different animals and/or suspensions. Therefore, the means for all the controls in the metformin ($n = 18$) and vanadium ($n = 14$) experiments contain a larger sample size (n) of animals and/or suspensions than all except the 1.0 mM metformin dose, which was studied in every experiment. Thus, when the number of wells with a particular concentration of metformin or vanadium differed from the total number of control wells (e.g., 0.5 mM metformin; $n = 9$), each result was compared only to that obtained in a corresponding control well from the same suspension in a pair-wise comparison. To examine the relationships between the medium concentrations of metformin or vanadium, glucose uptake, lactate production, and glucose conversion to lactate, and leptin secretion, simple and multiple linear regression analyses were performed with a statistics software package (StatView for Macintosh, Abacus Concepts, Inc., Berkeley, CA).

Because metformin exhibited toxic effects on adipocyte metabolism at concentrations greater than 5.0 mM, results from cultures incubated with metformin at concentrations greater than 5.0 mM were not included in these analyses. The relationship between lactate production from glucose and leptin secretion was also examined within the control groups alone. Data are expressed as means + SEM.

Results

Effects of Metformin

The effects of metformin on glucose uptake, lactate production, and leptin secretion were examined. Metformin at a concentration of 0.1 mM did not affect glucose uptake compared with the corresponding control suspensions containing insulin (0.16 nM) alone. At 0.25 mM, glucose uptake was increased ($\Delta = +9 \pm 8\%$), but the effect was not statistically significant perhaps due to the smaller number of trials ($n = 5$) performed at this concentration. Metformin stimulated glucose uptake at concentrations of 0.5 mM ($+37 \pm 10\%$, $p < 0.005$) and 1.0 mM ($+62 \pm 8\%$, $p < 0.0001$) compared with that in the corresponding control suspensions (Table 1, Figure 1). At 5.0 mM, glucose uptake was not significantly different from control. Higher concentrations of metformin (≥ 25.0 mM) markedly inhibited glucose uptake most likely reflecting a toxic effect of metformin at these very high concentrations. Metformin had no significant effect on lactate production at concentrations lower than 0.5 mM but increased lactate production at concentrations of 0.5 mM ($+105 \pm 43\%$, $p < 0.025$), 1.0 mM ($+186 \pm 31\%$, $p < 0.0001$), and 5.0 mM ($+202 \pm 52\%$, $p < 0.0025$) vs. insulin alone (Table 1, Figure 1). At concentrations of 25.0 mM, lactate production was markedly inhibited ($p < 0.0001$), because glucose utilization was

almost completely suppressed. Concentrations of metformin of 0.5 mM and below did not affect the proportional conversion of glucose to lactate (Table 1). However, glucose conversion to lactate was increased at a concentration of 1.0 mM, and this effect was marked at 5.0 mM with more than twice the amount of glucose released as lactate (Table 1). Although 1.0 mM metformin did increase mean glucose uptake over control rates, a significantly larger proportion of the glucose that was taken up was released as lactate. The concentration of 0.5 mM was the only level of metformin that induced a significant increase of glucose utilization without increasing the proportion of glucose carbon released as lactate (Table 1).

At concentrations of metformin lower than 0.5 mM, leptin secretion was unaffected. With metformin at 0.5 mM, the area under the curve (AUC) for leptin secretion over 96 hours was significantly greater ($+20.5 \pm 9\%$, $p < 0.05$) than control (Figure 2). Metformin inhibited leptin secretion at concentrations of 1.0 mM ($-25 \pm 8\%$, $p < 0.005$), 5.0 mM ($-89 \pm 4\%$, $p < 0.0001$), and by 90% at toxic concentrations of 25.0 mM ($p < 0.0001$) (Figure 2).

Within the 18 control wells, leptin secretion was inversely related to the conversion of glucose to lactate ($r = -0.61$; $p < 0.01$). At metformin concentrations from 0 to 5.0 mM, leptin secretion was inversely proportional to the log of the metformin concentration ($r = -0.53$; $p < 0.0001$), to lactate production ($r = -0.53$; $p < 0.0001$), and to the proportional conversion of glucose to lactate across metformin doses ($r = -0.74$; $p < 0.0001$) (Figure 3) but was not related to glucose uptake ($r = 0.13$; $p = 0.27$) by simple regression. By multiple regression analysis, leptin secretion was inversely related to the conversion of glucose to lactate ($p < 0.0001$) but not to the log of the metformin concentration ($p = 0.91$), lactate production ($p = 0.39$), or glucose uptake ($p = 0.62$). Leptin secretion was only increased over control by metformin at 0.5 mM, which was also the only concentration that significantly increased glucose uptake without shunting a greater proportion of the glucose into lactate production (Table 1). A similar inverse relationship ($r = -0.73$; $p < 0.0025$) between leptin production and anaerobic glucose metabolism to lactate was observed in 32 control wells containing either no insulin or a low insulin concentration of 0.16 nM (~ 20 $\mu\text{U/mL}$) (Figure 4).

Effects of Vanadium

The effects of vanadium on glucose uptake, lactate production, and leptin secretion were examined in adipocytes cultured with concentrations of vanadium of 0 to 50 μM . Vanadium at 5.0 μM ($+20 \pm 7\%$, $p < 0.01$), 10.0 μM ($+38 \pm 12\%$, $p < 0.02$), and 20.0 μM ($+34 \pm 13\%$, $p < 0.02$) increased glucose uptake, compared with rates of glucose uptake in the corresponding control suspensions (Table 2, Figure 5). The effect of vanadium at these con-

Table 1. Effects of metformin in the presence of 0.16 nM insulin on glucose uptake, lactate production, and the percentage of glucose carbon taken up that was released as lactate by isolated rat adipocytes over 96 hours in culture (mean \pm SEM)

[Metformin] (mM) + Insulin (0.16 nM)	Glucose uptake (μ mol) over 96 hours	Lactate production (μ mol) over 96 hours	Glucose to lactate (%)
Control (n = 18)	7.5 \pm 0.7	5.7 \pm 0.5	40.9 \pm 3.6
0.1 (n = 4)	6.5 \pm 0.9	5.9 \pm 0.6	47.2 \pm 6.3
0.25 (n = 5)	11.0 \pm 1.4	8.6 \pm 1.3	40.2 \pm 5.9
0.5 (n = 9)	11.0 \pm 1.2†	9.5 \pm 1.2†	44.9 \pm 5.1
1.0 (n = 18)	11.6 \pm 0.7‡	14.4 \pm 0.9‡	63.8 \pm 3.4*
5.0 (n = 15)	8.3 \pm 0.6	14.4 \pm 1.0‡	85.6 \pm 4.1‡

* $p = 0.01$; † $p = 0.005$; ‡ $p = 0.0005$; vs. corresponding controls from the same adipocyte suspensions.

concentrations was comparable to that of insulin at 1.6 nM, which increased glucose uptake by $38 \pm 8\%$ ($p < 0.0001$) (Table 2, Figure 5). Vanadium at 50.0 μ M did not significantly affect glucose uptake ($\Delta = -4 \pm 14\%$). Vanadium at 5.0 μ M increased lactate production by $17 \pm 8\%$ ($p < 0.025$). Mean lactate production in the six wells that served as controls for the 10.0 μ M concentration of vanadium was lower than average; however, lactate production was increased in five of six corresponding experimental wells. Thus, vanadium at 10 μ M increased lactate production by $61 \pm 20\%$ ($p < 0.02$) despite absolute lactate production being similar to the mean of the total 14 control wells. At 20.0 and 50.0 μ M, lactate production was not significantly different from that of the control (Table 2).

Insulin at 1.6 nM increased leptin secretion over 96 hours by $59 \pm 15\%$ ($p < 0.005$) and the 0- to 96-hour AUC by $38 \pm 8\%$ ($p < 0.0001$) (Figure 5). Leptin secretion was unaffected by vanadium at a concentration of 5 μ M. Higher concentrations of 10, 20, and 50 μ M consistently inhibited leptin secretion over 96 hours by $-33 \pm 6\%$ ($p < 0.0025$), $-53 \pm 7\%$ ($p < 0.0001$), and $-61 \pm 8\%$ ($p < 0.001$), respectively (Figure 6). Across vanadium concentrations, leptin secretion at 96 hours was positively correlated with glucose uptake ($r = 0.35$; $p < 0.02$) and inversely related to the log of the vanadium concentration ($r = -0.44$; $p < 0.0001$), to lactate production ($r = -0.30$; $p < 0.025$), and to the conversion of glucose to lactate ($r = -0.58$; $p < 0.0001$).

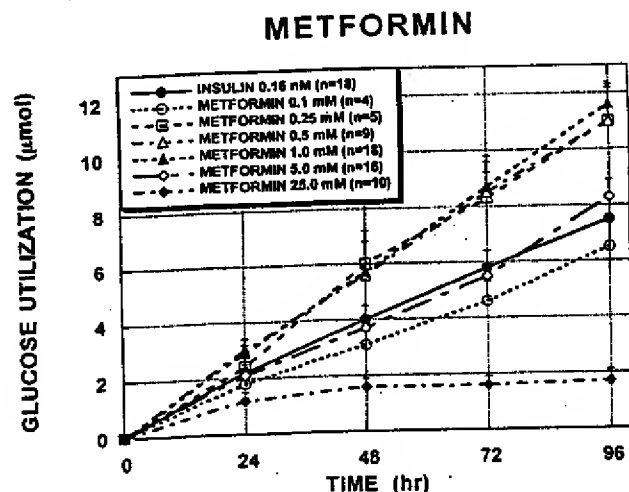


Figure 1. Glucose utilization (corrected for media sampling and replacement) over 96 hours by isolated rat adipocytes in primary culture with insulin at 0.16 nM and metformin at concentrations from 0 to 25.0 mM.

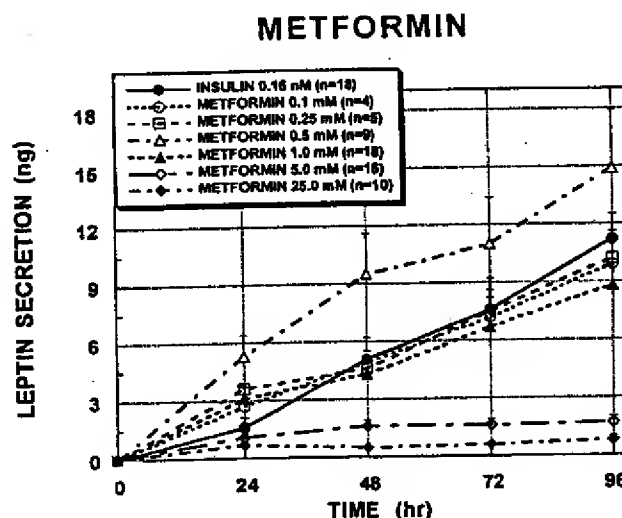


Figure 2. Leptin secretion (corrected for media sampling and replacement) over 96 hours by isolated rat adipocytes in primary culture with insulin at 0.16 nM and metformin at concentrations from 0 to 25.0 mM.

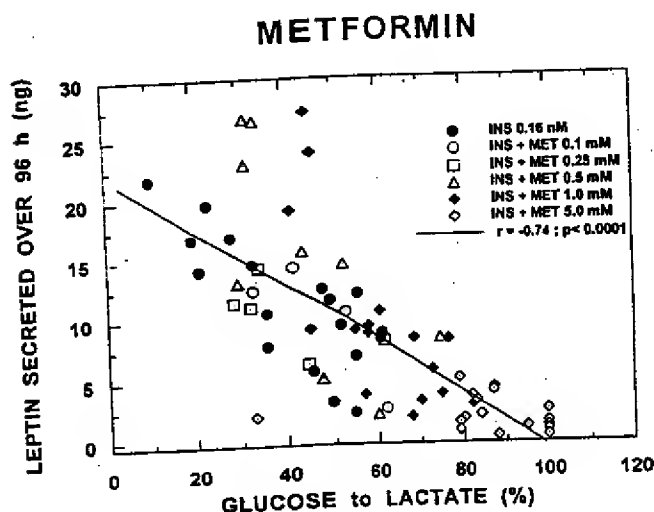


Figure 3. Relationship between the percentage of glucose taken up and released as lactate and leptin secretion over 96 hours by adipocytes in primary culture with insulin (INS) at 0.16 nM and metformin (MET) at concentrations from 0 to 5.0 mM. Leptin secretion, glucose utilization, and lactate production are corrected for media sampling and replacement.

0.0001) (data not shown). By multiple regression analysis, leptin secretion at 96 hours was more closely related to glucose conversion to lactate ($p < 0.0001$) than to absolute lactate production ($p < 0.02$) or the log of the vanadium concentration ($p < 0.005$) and was not related to absolute

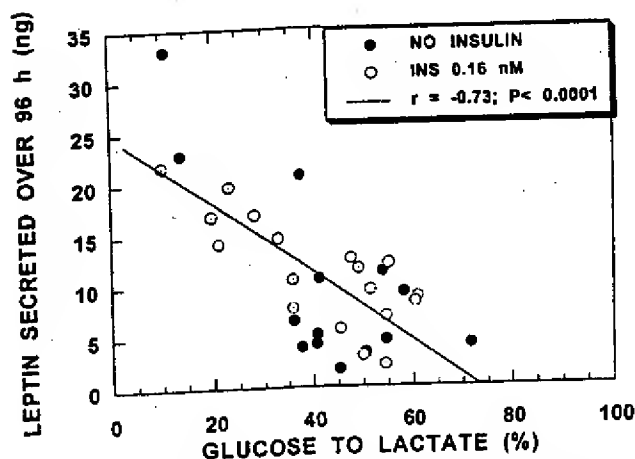


Figure 4. Relationship between the percentage of glucose taken up and released as lactate and leptin secretion over 96 hours by adipocytes in primary culture in 32 control wells containing no added insulin or insulin at a low concentration of 0.16 nM. Leptin secretion, glucose utilization, and lactate production are corrected for media sampling and replacement.

glucose uptake ($p = 0.22$). Despite the relationship between leptin secretion and the conversion of glucose to lactate across vanadium concentrations, unlike with metformin, the percentage of glucose released as lactate was not altered by any concentration of vanadium (Table 2). In contrast, insulin at 1.6 nM, which significantly decreased the proportional conversion of glucose to lactate (i.e., anaerobic glucose metabolism) (Table 2), stimulated leptin secretion (Figure 6).

Discussion

We have recently reported that insulin-mediated glucose metabolism is an important factor regulating leptin expression and secretion in isolated rat adipocytes (28). Some previous studies have shown that drugs in the thiazolidinedione class, which are used in the treatment of type 2 diabetes, can inhibit leptin production in vitro and in vivo (38,39). In the present study, we examined the effects of metformin and vanadium, two other antidiabetic drugs known to increase cellular glucose utilization, on leptin secretion, glucose uptake, and lactate production in isolated cultured rat adipocytes. Our goal was to use these compounds as tools to examine their effects for altering adipocyte glucose utilization on leptin secretion. Therefore, we used concentrations within a range that were found to produce significant increases of adipocyte glucose uptake. Particularly in the case of metformin, these concentrations (0.25 to 5.0 mM) were far above the range of plasma concentrations (0.005 to 0.02 mM) observed in patients treated with therapeutic doses of metformin (40). In fact, at therapeutic concentrations, metformin seems to act primarily to inhibit hepatic glucose production with limited, if any, effects on peripheral glucose uptake (41–43). At therapeutic concentrations, metformin generally has little direct effect on glucose utilization in vitro (44,45). At concentrations higher than those achieved in serum with therapeutic metformin administration, metformin stimulates glucose transport by rat (46,47) and human adipocytes (48), and in rat and human skeletal muscle (see reviews in 29,34,48,49). At the cellular level, high concentrations of metformin increase insulin receptor binding, along with tyrosine kinase activity, glucose transport, and glycogen synthesis (33,34).

In the present study, metformin concentrations ranging from 0.5 to 5.0 mM increased both glucose uptake and lactate production. In addition to increasing absolute lactate production, metformin at 1.0 and 5.0 mM increased the percentage of glucose carbon that was metabolized to lactate and released into the culture media by 80% to 170%. At high concentrations of metformin ($= 25.0$ mM), both glucose uptake and lactate production were markedly inhibited, most likely due to a toxic effect of very high levels of metformin on cellular metabolism. Metformin at 0.5 mM modestly increased leptin secretion by $\sim 20\%$.

Table 2. Effects of insulin (1.6 nM) or vanadium on glucose uptake, lactate production, and the percentage of glucose carbon taken up that was released as lactate by isolated rat adipocytes over 96 hours in culture (mean \pm SEM)

[Vanadium] (μ M); no insulin added	Glucose uptake (μ mol) over 96 hours	Lactate production (μ mol) over 96 hours	Glucose to lactate (%)
Control ($n = 14$)	6.8 ± 0.5	5.6 ± 0.6	42.4 ± 4.3
1.6 nM Ins ($n = 14$)	$9.4 \pm 0.9\%$	5.7 ± 0.7	$33.0 \pm 3.5\ddagger$
5.0 ($n = 12$)	$7.8 \pm 1.1\%$	$5.7 \pm 0.6^*$	40.5 ± 4.7
10.0 ($n = 6$)	$8.2 \pm 1.3\ddagger$	$5.2 \pm 0.8\ddagger$	36.0 ± 6.5
20.0 ($n = 12$)	$8.7 \pm 1.2\ddagger$	6.3 ± 0.6	41.6 ± 5.0
50.0 ($n = 13$)	6.9 ± 1.02	4.9 ± 0.5	53.3 ± 9.4

* $p = 0.05$; $\ddagger p = 0.02$; $\ddagger p = 0.0025$; $\%$ $p = 0.0005$; vs. corresponding control wells from the same adipocyte suspensions.

Importantly, this was the only concentration of metformin tested that increased glucose uptake without shunting a greater proportion of glucose into lactate production. In contrast, at concentrations of 1.0 mM and higher, leptin secretion was modestly to markedly suppressed.

A significant proportion of glucose taken up by adipose tissue is metabolized to lactate and released (50). At metformin concentrations ≤ 5.0 mM, leptin secretion was inversely related to the amount of glucose taken up by the adipocytes, converted to lactate, and released into the media. We have previously observed that the stimulation of leptin secretion by insulin is associated not only with increased glucose utilization, but with a decrease in the pro-

portional conversion of glucose to lactate (28), a finding that was also observed within the control groups in the present study (Figure 4). Thus, when lactate production is increased, less carbon derived from glucose is available to enter the tricarboxylic acid cycle either for oxidation or use in de novo lipogenesis. Together, these data suggest that it is not glucose uptake, per se, but its metabolism beyond pyruvate and lactate in the adipocyte that is involved in the action of glucose to stimulate leptin secretion. Thus, the anaerobic metabolism of glucose does not stimulate leptin production. The entry of glucose into the hexosamine biosynthetic pathway and the production of UDP-glucosamine have been suggested as a mechanism by which glucose utilization can

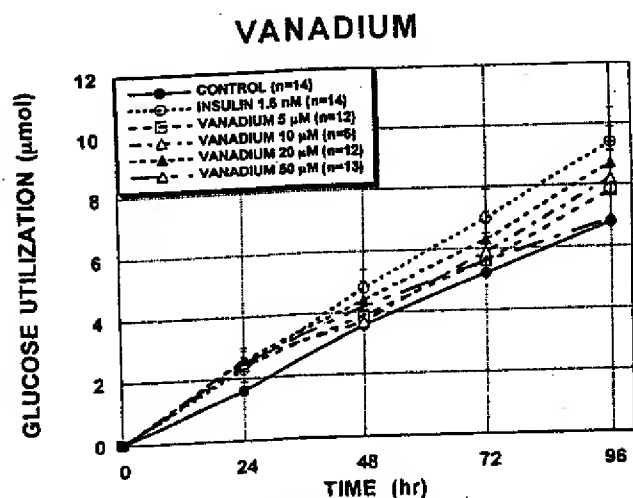


Figure 5. Glucose utilization (corrected for media sampling and replacement) over 96 hours in by isolated rat adipocytes in primary culture with vanadium at concentrations from 0 to 50.0 μ M or with insulin at 1.6 nM.

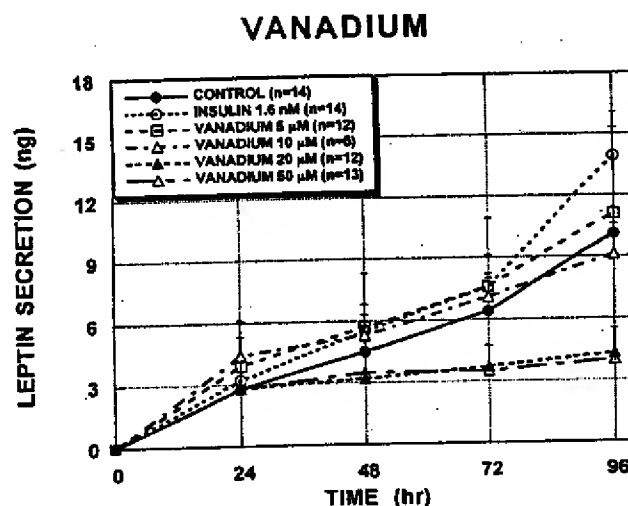


Figure 6. Leptin secretion (corrected for media sampling and replacement) over 96 hours by isolated rat adipocytes in primary culture with vanadium at concentrations from 0 to 50.0 μ M or with insulin at 1.6 nM.

stimulate leptin production in adipose tissue (51). However, our results indicate that glucose can be metabolized to lactate, a point well beyond where glucose enters the hexosamine pathway, without stimulating leptin production. Therefore, if the hexosamine pathway were to be the sole mechanism by which glucose regulates leptin production, one would need to postulate that either anaerobic glucose metabolism and/or metformin itself, have an inhibitory effect on glucose entry into this pathway. The results of the present study suggest that it is more likely that the effect of glucose metabolism to stimulate leptin production involves glucose oxidation and/or the production of lipogenic precursors.

Metformin at 0.1 and 0.25 mM did not effect glucose metabolism or leptin secretion. Thus, metformin at therapeutic levels is unlikely to affect leptin production *in vivo*. Of the concentrations of metformin tested in this study, only 0.5 mM increased glucose uptake without shunting a larger proportion of the glucose into lactate. As previously discussed, this was the only concentration of metformin that had effects on glucose metabolism that did not inhibit leptin secretion, and, in fact, leptin secretion was modestly increased at 0.5 mM. Thus, only when glucose uptake and its metabolism beyond lactate were simultaneously increased, did we observe an increase, and not an inhibition, of leptin secretion. Therefore, it seems that only within a very narrow concentration range is metformin able to have a net effect to increase glucose uptake as well as its metabolism beyond lactate in isolated adipocytes. Accordingly, the effects of metformin to inhibit leptin secretion at most concentrations examined is likely to be a result of its effects to direct pyruvate metabolism into lactate and away from other potential pathways for pyruvate metabolism such as oxidation or lipogenesis.

The use of vanadium-containing compounds in the treatment of diabetes has been widely investigated in animals (52,53), and a few clinical trials have been conducted in human patients (54,55). A compound structurally similar to the form of organified vanadium used in this study (BMOV) has recently entered Phase I clinical trials. To our knowledge, the present study is the first report examining the effects of a vanadium compound on leptin production *in vivo* or *in vitro*.

Vanadium stimulated glucose uptake at concentrations up to 20 μM , whereas glucose uptake was not affected by a concentration of 50 μM . Lactate production was modestly increased at the lower concentrations of vanadium. We found that vanadium at a low concentration of 5.0 μM did not affect leptin production, however, concentrations of 10.0 μM and higher inhibited leptin secretion from isolated adipocytes by 30% to 60%. Although the amount of leptin secreted was inversely proportional to the percentage conversion of glucose to lactate across the concentrations of

vanadium tested, this relationship was significantly weaker than that observed across metformin concentrations.

Furthermore, the proportion of glucose taken up and released as lactate was unaffected by vanadium at any concentration. Thus, in contrast to what was observed with metformin, the ability of vanadium to inhibit leptin secretion seems to be independent of any effects on glucose metabolism or lactate production, most likely because it does not increase the proportion of glucose fluxing into anaerobic metabolism.

The observed effects of vanadium result from one or more of the multiple known biological actions of vanadium in cells. These include the inhibition of protein tyrosine phosphatases and the activation of cytosolic protein-tyrosine kinases, resulting in the alteration of cellular tyrosine phosphorylation content (30,56). Vanadium has also been shown to exert direct inhibitory effects on a number of other cellular enzymes, including acid, alkaline, and dual-function phosphatases, ATPases, glucose-6-phosphatase, and fructose-2,6-bisphosphatase (30,55). At high concentrations, vanadium might exert some toxic effects on the cells, an effect which could underlie the lack of effect of the highest concentration of vanadium to stimulate glucose uptake, as well as the inhibition of leptin production at the two highest concentrations examined. In particular, the effects of vanadium to inhibit the activity of one or more enzymes involved in cellular energy metabolism could both inhibit leptin production and, at high concentrations, impair the ability of the cell to utilize energy derived from glucose metabolism.

In vanadium-treated animals, plasma vanadium concentrations have been estimated to be in the 10 to 20 μM range and in human clinical trials in the 1 to 5 μM range (53). Although it is unlikely based on the present results that the concentration of vanadium achieved in humans would be sufficient to affect leptin production, previous human studies employed low doses of vanadyl sulfate or sodium metavanadate, which are molecular forms that exhibit poor bioavailability. The potential effects on leptin secretion of the more readily absorbed forms of vanadium, such as the organified vanadium compound (BMOV) used in the present study (31), should therefore be considered.

In summary, both metformin and vanadium inhibit leptin secretion from primary cultures of rat adipocyte at concentrations that significantly increase glucose utilization. The inhibition of leptin production by metformin, but not by vanadium, is related to an increased conversion of glucose to lactate (i.e., anaerobic metabolism). This effect of metformin, coupled with our previous findings (28), suggests that the effect of glucose utilization to stimulate leptin production is not mediated by glucose uptake *per se* but involves the metabolism of glucose beyond pyruvate to a fate other than lactate, possibly oxidation or lipogenesis. Thus, metformin is a useful tool for examining the effects of

increasing anaerobic glucose metabolism. Further research, including examination of the potential roles of glucose oxidation and lipogenesis, needs to be conducted to determine the precise biochemical and molecular mechanisms by which glucose metabolism regulates leptin production.

Acknowledgments

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